Arabinoxylan-degrading enzymes of the wheat pathogen Stagonospora nodorum

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ARABINOXYLAN-DEGRADING ENZYMES OF THE WHEAT PATHOGEN *Stagonospora nodorum*

submitted by

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Dedication

I would like to dedicate this thesis to my sister, Adriana, in her struggle to overcome all the side effects she had after her brain tumour operation.

May God Bless Her.

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ABSTRACT

Penetration and colonization by *Stagonospora nodorum*, the causal agent of leaf and glume blotch of wheat, may involve enzymic degradation of host barriers; appressorial structures are not consistent feature of penetration sites and host cell walls are altered ultrastructurally. To test this, a study of the cell wall-degrading enzymes (CWDE) produced by the fungus at the early stages of infection was carried out, and compared with the depolymerase and glycosidase activities produced *in vitro* on minimal medium with wheat cell wall as sole source of carbon source. Enzyme activities produced *in vivo*, were determined from inoculation drops after inoculation of detached wheat leaves with spores, from extracts from infected leaves and from apoplastic fluids (intercellular washing fluids or IWF). Under these conditions, the fungus produced a cocktail of extracellular hydrolases and proteases. The major CWDEs secreted by *S. nodorum* were xylanases and arabinosidases; this possibly reflects adaptation of the fungus, as with other pathogens of cereals, to the composition of the cell wall of *Gramineae* in which arabinoxylans are the predominant matrix polymer(s). Endo-1, 4-β-xylanases catalyse the endo-hydrolysis of xylosidic linkages and α-arabinofuranosidase the removal of appendant arabinose for the efficient degradation of substituted xylans. Both *in vitro* and during early infection there was rapid production of xylanase and arabinosidase. Activities were first detected on leaf surfaces after 24 h, which is before penetration. Also there was some correlation of the levels of activity of these enzymes with the aggressiveness of the three isolates used in this study. Isoelectric focusing (IEF) in thin acrylamide-urea gels were used to resolve xylanase and arabinosidase isoforms. Two arabinosidase isoforms (pI's 4.5 and 3.9) and two xylanase isoforms (pI's 4.5 and 4.0) of likely fungal origin were present in the IWF of inoculated plants, in inoculating droplets and the culture filtrate. Difficulties arose from the presence of sugars, which caused proteins to aggregate into high molecular weight species; also glycosylated protein complexes charged with sialic acids were eventually resolved through gel filtration and deglycosylation both under mild denaturing conditions. It was not possible to identify clearly if the enzymes within the aggregate were multifunctional proteins or multienzymatic complexes, since purification of single protein bands with single

activities was not achieved even with diverse purification techniques. However, it was possible to isolate a putative arabinosidase-xylanase complex by affinity chromatography in cross-linked substrates and gel filtration under mild denaturing conditions. The putative complex appeared to be glycosylated and contained 47% (w/w) sugars of which 8% (w/w) were sialic acids. To the knowledge of the author this is only the second report of sialic acids in a plant pathogenic fungus. Urea gradient gels suggested that in the putative affinity-purified complex at least one protein is a bifunctional arabinosidase xylanase but it was not possible to obtain definitive biochemical evidence telling how they associate. From databases, DNA and RNA strategies were developed to increase the knowledge of these CWDE in *S. nodorum*. A collection of protein and nucleotide sequences of fungal and bacterial xylanases and arabinosidases were aligned and two regions of high similarity in the amino acid and nucleotide sequences were identified. Oligonucleotides were designed based on these regions and used as primers to PCR-amplify DNA segments from genomic DNA of 5 isolates of the fungus. The PCR-amplified probes also hybridized to a small number of restriction fragments under high stringency Southern blot analyses suggesting a family of genes and related sequences in the *S. nodorum* genome. A cDNA library prepared from *S. nodorum* grown in basal medium containing wheat cell walls as the sole carbon source was screened and two xylanase and three arabinosidase cDNAs with different 3' untranslated ends were identified and sequenced in both strands. Both xylanases contained Family 11 glycosyl hydrolases catalytic domains. Arabinosidases were all Family 62 glycosyl hydrolases. Clones did not appear to have a modular structure as no cellulose-binding domain (CBD) or xylose-binding domain (XBD) was identified in the deduced amino acid sequences. The evidence for multiple xylanase and arabinosidase genes in *S. nodorum* corroborates the biochemical evidence for multiple isoforms of these enzymes. The evidence presented however confounds the immediate possibility of exploiting gene knockout mutants for confirming the role of these enzymes during infection. This study can be considered as a preliminary result towards identification of specific members of the xylanase and arabinosidase gene families expressed during different stages of infection and their temporal pattern of expression that is required to understand the role of these enzymes as determinants of pathogenicity.

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CHAPTER 1 GENERAL INTRODUCTION

1. 1 *Stagonospora nodorum*

Stagonospora nodorum. Berk. (syn. *Septoria nodorum*), telomorph *Phaesphaeria nodorum* (syn. *Leptosphaeria nodorum*) is the causal agent of leaf and glume blotch. It occurs principally on wheat but also on barley and rye. Most isolates of *S.nodorum* are specialised to either wheat or barley (Osbourn *et al.*, 1986).

Septoria nodorum is a widely distributed ascomycete fungus, reported to occur in many parts of Europe, North and South America, Africa, Asia, Australia and New Zealand. From surveys of winter wheat in England and Wales estimates of annual national losses caused by the fungus range from 0.1 to 7.4%. In Scotland it was estimated that the losses in the yield per year were 3.1% and 6.5% in wheat (King *et al.*, 1983).

The disease increased in importance from 1975-1985 reflecting increased wheat growing in areas with cool damp summers and mild winters. The increased use of dwarf varieties has favoured disease by reducing the distance that spores have to travel by rain splash and hence more of the plant becomes infected. Non-ploughing techniques have also increased disease incidence because failure to remove crop debris provides another inoculum source. From 1978-1982 and again in 1986, *S. nodorum* was the most important foliar disease of winter wheat in the UK (Polley and Thomas, 1991).

Genetic studies on the different strains have shown that there is considerable divergence at the DNA level among strains but this was not related to host specificity. *S. nodorum* is highly variable on a microgeographical scale, with a large number of different genotypes present within a single infected field, and even within small areas

of that field. Populations of this fungus are polymorphic not only for molecular markers, which are generally assumed to be neutral with respect to fitness, but also for phenotypic characters that are likely to be subject to selection Karyotype studies showed that the genome contains around 30Mb of DNA organised into 14-19 chromosomes ranging from 0.4 to 3.5 Mb in size. There was no correlation between strain karyotype and host adaptation (Caten and Newton, 2000).

Septoria nodorum is also capable of infecting perennial grasses, which may allow the pathogen to survive between wheat crops (Krupinsky, 1997a). Successive passage of these grass isolates through wheat failed to increase their aggressiveness or adapt them to wheat, suggesting that generally these isolates are unlikely to cause severe symptoms on wheat (Krupinsky, 1997b).

1. 1. 1 Disease symptoms

Stagonospora nodorum infection of seedlings results in twisted, distorted and stunted plants. On mature plants the disease affects all aerial parts, the leaves showing symptoms about 10 days after infection. On mature leaves the first symptoms are small necrotic lesions, which later become golden brown and badly infected plants often appear ginger in colour. The shape of the lesions is variable but is often elliptical and surrounded by a darker, slightly purplish margin .The leaf spots eventually coalesce, resulting in large areas of dead, dry, sometimes split tissue. Within this tissue pycnidia form and spore masses (cirri) are released during wet weather. The tissue appears salmon-pink in colour (Zinkernagel *et al.*, 1988).

The nodes of the culm and rachis are also often attacked and become purplish-black and also bear pycnidia. Leaf infections have been shown to cause reductions in yield, however the greatest losses occur from infections of the heads, resulting in drastic reductions in grain size. Such infection of the glumes can be extensive, the dark, purplish-brown lesions spreading downwards from the tip. Pycnidia are produced in these lesions and in cases of severe infection, on almost all of the glumes. Symptom

expression will depend on isolate, host cultivar and environment (Karjalainen, 1985).

Reports of factors affecting infection and symptom expression include the influence of humidity, temperature, light and spore load. Formation of pycnidia of *S. nodorum* on detached leaves occurs over a range of temperatures between 18 and 24 °C. All reports have indicated a requirement for light and senescing tissue before initiation of pycnidia. Greater production of pycnidia and spores occurred on straw of a susceptible than resistant cultivar. The hyphal growth on the surface of leaves was similar on resistant and susceptible cultivars and no differences have been found in the germination and penetration of spores. However, hyphal development within the leaf is much less in resistant cultivars (King *et al.*, 1983).

1. 1. 2 Disease cycle

Stagonospora nodorum overwinters as dormant mycelium, pycnidia and pseudothecia on seed, stubble, debris, autumn sown crops and volunteers. Initial infection in the autumn or spring, in the absence of crop debris can result from wind-borne ascospores released from pseudothecia, particularly because ascospores can germinate and infect when temperatures are lower than 5 °C, while pycnidiospores (asexual spores) required temperatures above 5 °C to infect leaves. As temperatures rise and humidity increases, asexual pycniospores are produced from pycnidia. These are splash-dispersed vertically up the plant and horizontally from plant to plant. Periods of rain are essential for spore dispersal, while temperatures of 20-27 °C together with 100% humidity are optimal for spore germination. Under these conditions the disease cycle can be completed in 10-14 days (Bird and Ride, 1981).

Survival of the imperfect state on stubble and debris from previous crops is the most important means of perpetuation of *S. nodorum* between successive crops, even though, in glasshouse tests, *S. nodorum* can establish itself on tissue of several different dicotyledonous cultivated and wild plants. The perfect states of the pathogen can also be important in the carry-over of the disease in crop debris. In addition to

this, seed infection is a probable source of transmission from one crop to the next, not only in wheat but also in barley (King *et al.*, 1983).

It has been stated that true resistance to *S. nodorum* had not been shown to exist, although a degree of tolerance may occur, but that heritable resistance to *S. tritici* has been clearly demonstrated. The precise significance of the terms resistance and tolerance as used in reports is not always clear. Resistance is often used to indicate relatively less severe disease due to any cause. Tolerance, in the sense of a relatively small yield reduction arising from relatively severe infection, has been reported for *S. nodorum* (Caten and Newton, 2000). Susceptibility is seen as an increased number of lesions which eventually coalesce to form diffuse lesions with less discrete margins, surrounded by large chlorotic areas. Tolerant cultivars develop fewer and /or smaller lesions and show retention of green leaf colour rather that prevention of infection (King *et al.*, 1983).

Resistance of wheat to *S. nodorum* is polygenic; suggesting a number of mechanisms may be involved, possibly acting at different stages of infection. One suggested resistance response that has been studied extensively is the lignification observed in response to penetration and growth of *S. nodorum* through the whole plant. No effects of variety resistance on germination or number of appressoria produced have been observed. However, germ tubes on resistant varieties may be shorter. Lignification as a general defence response may act as a mechanical barrier to hyphae, protecting the host cell wall from enzymatic attack or restricting the diffusion of water, nutrients and toxins (Caten and Newton, 2000; Bird and Ride, 1981).

Interactions of *S. nodorum* with other pathogens can either increase or decrease disease severity. Saprophytes as well as pathogens may affect the disposition of wheat tissue to infection. Several phylloplane organisms have been shown to predispose wheat tissue to infection but the opposite situation, where disease severity is diminished by the presence of antagonistic saprophytes, has also been demonstrated (Dewey *et al.*, 1999)

Several research groups currently use *Stagonospora nodorum* as a model pathogen. This choice is due to factors such as relative easy of handling and genetic tractability besides its importance as a major pathogen of wheat and barley and a possible representative of fungal pathogens of cereals (Dancer *et al.*, 1997).

1. 2 Host-Pathogen Interactions

Plants should recognise microorganisms as pathogens or symbionts, as the microorganisms should recognise the plant species, with which they establish specific relations. The specificity of the plant-microbe interaction is the result of mutual recognition accomplish by macromolecules present in both organisms. The result of the interaction between host and pathogen can be either compatible or incompatible depending on whether there is microbial colonisation, development of symptoms and disease or just limited microbial growth, lack of symptoms and often development of localised necrotic lesion referred as hypersensitive response. Fungal phytopathogenicity is presumed to be dependent upon pathogen factors required for penetration, survival, symptom development and growth within the plant host (Coleman *et al.*, 1997; Vidhyasekaran, 1988).

Fungal infection usually starts with the spore germination in the proximity of the host surface. Many phytopathogenic fungi penetrate *via* plant stomata, lenticels and wounds whereas others attack their host by direct penetration through the cuticle. Also, fungi take many different physical routes during colonisation of plants, including the intercellular spaces, xylem, within cell walls, through cell walls and therefore direct contact with the plant cell membrane. The different routes taken by fungi during colonisation of plants and the sites at which fungal growth may be restricted in resistant plants are indicated in Fig 1 (Oliver and Osbourn, 1995; Mansfield, 1990).

In order for a fungus to colonise a plant it must overcome potential mechanisms of resistance. This may be achieved by failing to provoke a response or by detoxifying

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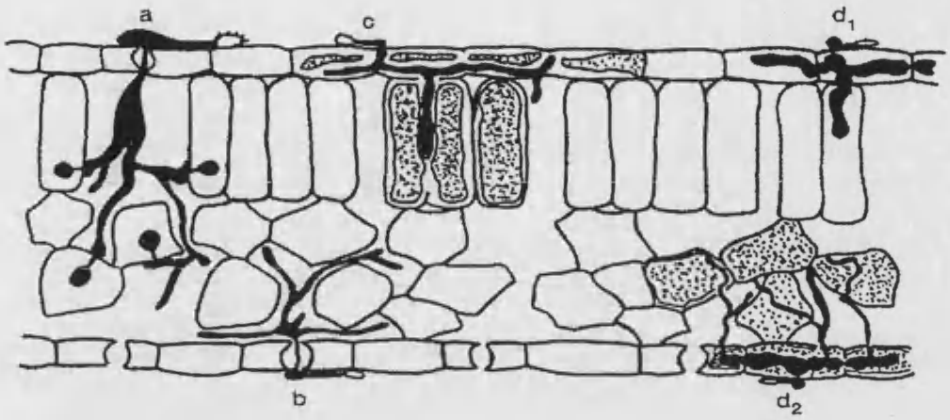

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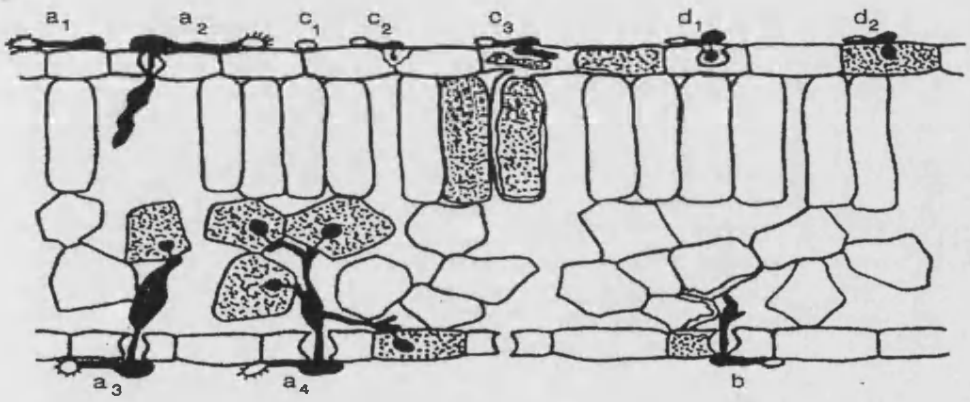


Figure 1:

(I) Routes taken by pathogenic fungi: a) biotrophic rust fungus, stomatal penetration and production of intercellular hyphae and intercellular haustoria, b) biotrophic intercellular growth of *Cladosporium fulvum*, c) necrotrophic *Botrytis sp.*, direct penetration into epidermal cells, growth of intramural and intercellular hyphae within necrotic tissue, d) the temporary biotroph *Colletotrichum lindermuthianum*; the initial phase of bitrophic intracellular growth of large primary hyphae (d1) is followed in the necrotrophic phase(d2) by the rapid intra- and intercellular growth of secondary hyphae. Dead plant cells are shaded.

(II) Sites of restriction of fungal development : a) inhibition of rust fungus on the leaf surface (a1), during formation of a sub-stomal vesicle (a2), and following the HR after production of the first rudimentary haustorium (a3) or at later stage of infection (a4); b) induction of the HR and cell wall atterations restricts *Cladosporium fulvum*; c) inhibition of *Botrytis sp.*,failure to germinate (c1), prevention of penetration by cell wall alterations (c2) or cessation of growth in a limited lesion (c3); d) colonisation by *Colletotrichum lindermuthianum* may be restricted by papilla formation (d1) or the HR (d2). Dead plant cells are shaded. Figure according to Mansfield (1990).

antimicrobial factors that may be produced. Possible routes to successful parasitism and the critical stages at which signalling associated with recognition events may determine the outcome of the plant /fungus interaction are shown in Fig 2 (Mansfield, 1990).

The cuticle and cell wall beneath together with other preformed antimicrobial compounds constitute the first barriers the pathogen encounter. Pathogenic fungi may employ three different methods to overcome these physical barriers: mechanical penetration, enzymatic penetration or enzyme-assisted mechanical pressure (Dean, 1997; Oliver and Osbourn, 1995). Mechanical penetration may involve formation of appressoria and melanin biosynthesis as Howard *et al.* (1991) have demonstrated in *Magnaporthe grisea.* Melanin is produced by several fungi in which the highest accumulation of this pigment occurs before penetration. It appears that the role of melanin is mediation of the build-up of hydrostatic pressure that allows the fungus to rupture the plant epidermis mechanically (Schafer, 1994). Many fungi however achieve penetration from appressoria in the absence of melanin formation.

The infection process in *S. nodorum* begins with germination of spores only 2 hours after inoculation. Preliminary studies on *S. nodorum* have been done with transmission and scanning electron microscopy of inoculated leaves. Immediately after germination the germ tubes of *S. nodorum* appeared to be surrounded by amorphous material, which in most cases, served to attach growing hyphae with the host surface (Karjalainen and Lounatmaa, 1986). Extensive mycelial growth on the leaf surface preceding penetration has always been observed for the isolates used in our group (Carlile *et al.*, 2000). Appressoria are very infrequent (Cooper personal communication) although Karjalainen and Lounatmaa (1986) described their production. Zinkernagel *et al.* (1988) had also observed extensive subcuticular growth. The cuticle appeared to be degraded by secretions (amorphous material) from the conidia and the hyphae emerging from the conidia. The extensive epiphytic phase and the penetration that results from it are poorly understood and warrant investigation. Clearly *S. nodorum* does not conform to most other fungal "models" in which appressorial timing is synchronised, rapid and predictable.

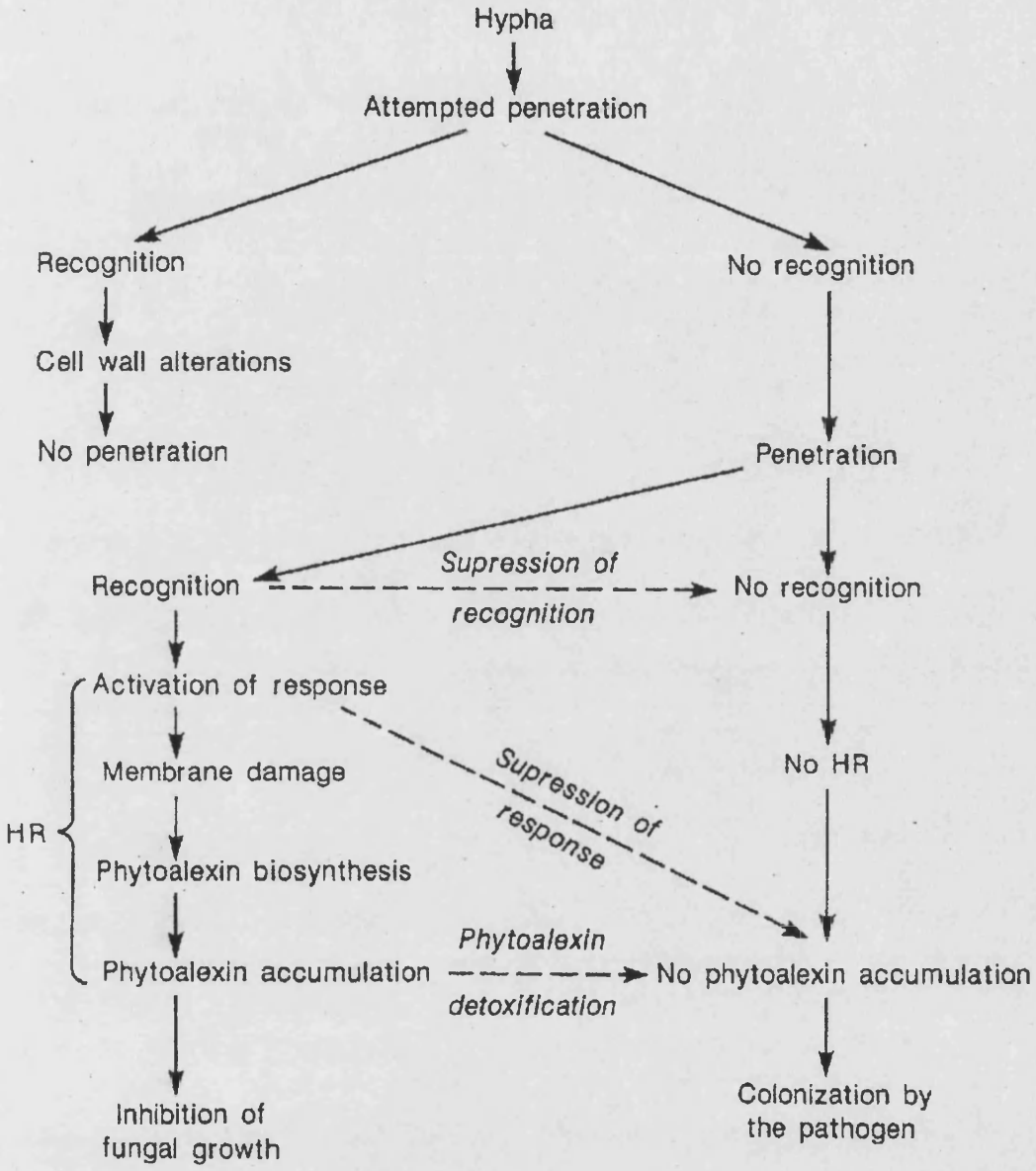


Figure 2: Possible routes and the critical stages at which signalling associated with recognition events may determine successful parasitism or recognition leading to resistance during plant/fungus interactions (Mansfield, 1990).

According to Karjalainen & Lounatmaa (1986), close contact between germ-tubes and their host surface, and attempted penetration directly through the cell wall walls were frequently observed at three days after inoculation. Regions beneath the penetration sites appeared more diffuse than the normal, suggesting that penetration occurred with enzymatic hydrolysis. Following penetration of the host, hyphae appeared to grow intercellularly again with apparent enzyme degradation of the cell wall, spreading slowly in the mesophyll. At this time, chloroplast disintegration was also apparent. These ultrastructural events could be correlated with marked changes in host plant physiology, such as chlorophyll breakdown, decrease of photosynthetic and respiration rate, alterations in nitrogen and carbohydrate metabolism and finally appearance of symptoms.

The mechanisms of pathogenicity of *Septoria nodorum* remain poorly understood but it is known to produce a wide spectrum of extarcellular CWDE (see below) and two putative toxins, septorine and mycophenollic acid, produced in culture but its production *in vivo* has not been demonstrated. Septorine has a decoupling action on mitochondria isolated from wheat coleoptiles while mycophenollic acid acts by directly inhibiting the Calvin cycle. No correlation between toxin production and pathogenicity has been established, although the toxins may produce necrotic symptoms, which occur in terminal stages in the field (Keller *et al.*, 1994).

Both saprophytic and plant parasitic fungi produce extracellular enzymes which can degrade the cell wall components of plants. These fungi not only digest plant cell wall polymers to obtain an important nutrient source but also to aid in penetrating cells and spreading through plant tissue (Annis and Goodwin, 1997). Considering that cell walls are one of the first structures to be contacted during host-pathogen interactions, it is not surprising that many successful pathogens require wall-degrading enzymes (CWDE) though it is known that some pathogens do not appeared to use them or even produce them, in the case of *Erwinia amylovora* (Cooper, 1983). *S. nodorum* when grown in liquid culture with host cell walls as main carbon source, secretes numerous extracellular CWDE (Carlile *et al.*, 2000; Lehtinen, 1993; Magro, 1984).

Major activities were xylanase, β-1,3-glucanase and protease. Activities detected *in vivo* were xylanase, arabinosidase and a trypsin-like protease.

Also it would seem to be a selective advantage to plants to recognise action of CWDE as signals of incipient attack. In that sense cell wall-degrading enzymes are considered to be examples of "basic compatibility factors", meaning that they are required by pathogens for infection but are not determinants of race or cultivar specificity (Walton, 1994).

This review will focus on the enzymatic penetration of fungal pathogens into their host and the consequent tissue maceration. Essential to the understanding of the mode of action of CWDE is the knowledge of primary cell wall structure and composition (Keon *et al.*, 1987), thus, there will follow a brief look at plant cell wall structure.

1. 3 Host Cell Wall

It is now known that the plant cell wall is a much more dynamic entity than previously imagined. It is considered a metabolically active compartment of the cell; the composition of the wall can change significantly as cells grow and walls also contain enzymes involved in a range of physiological functions (Carpita & Gibeaut, 1993).

In general, the cell wall is composed of three layers: the middle lamella, the primary cell wall and the secondary cell wall. All three layers are composed of two domains: a microfibrillar and a matrix domain. The microfibrilar domain is composed of cellulose and can be distinguished from the matrix by its relative homogeneity and its high degree of crystallinity. The matrix domain is heterogeneous in contrast, and can be composed of pectins, hemicellusoses proteins and phenolics (Smart, 1991).

The middle lamella or the "intercellular cement", occurs between adjacent cells and bind cells together, being the first layer to be synthesised at the cell plate at the time

of cell division and deposited as long as the cell is still expanding. The primary cell wall is the first formed wall, laid down by growing cells. The secondary cell wall is similar in structure to the former but tends to contain more cellulose giving it a more rigid structure (Smith, 1993).

According to Fry (1998), understanding the biochemistry of the primary cell walls is important because of the crucial roles it plays in determining (a) the rate and direction of cell expansion, (b) the tenacity of cell-bonding, (c) the susceptibility of the tissue to external enzymatic attack, (d) the water- and ion-binding capacity of the cell wall, and (e) the production of oligosaccharins (wall fragments with signalling roles).

Over the last two decades a great effort has been made in that direction. After many polysaccharides and protein components were characterised often with the use of highly-purified cell wall -degrading enzymes from plant pathogens, it has become clear that the cell wall composition in the grasses (gramineae) differs radically from that found in dicotyledonous plants (Carpita and Gibeaut, 1993; Cooper *et al.*, 1988). In general, the main structural compounds of the primary cell wall are:

i) Cellulose

Linear chains microfibrils, of 1→4 β-linked D-glucose condensed to form crystalline regions that wrap around each other, consisting around 20 - 30% of the primary cell wall, but they can compose over 40% of the secondary cell wall, where the microfibrils are arranged as parallel lamellae. Cellulose is stabilised by inter and intra-molecular hydrogen bonding between the linear chains (Fry, 1998; Carpita & Gibeaut, 1993).

ii) Hemicelluloses

Matrix polysaccharides of plant cell walls, are much more complex than cellulose and consist of heteroglycans containing different sugar residues. Among them are: noncellulosic β-glucans (β-1,3 and β-1,4 glucans), mannans, xylogucan, arabinans and xylans. In the cell walls of land plants, xylan is the most common hemicellulosic polysaccharide, representing more than 30 % of dry weight (Joselau *et al.*, 1992).

Xylans are composed of 1,4 -linked β-D-xylanopyranosyl residues. Most xylans occur as heteropolysaccharides, containing different substituents groups in the backbone and side chains. The most common substituents found are acetyl, arabinosyl, and glucuronosyl residues. Homoxylans on the other hand, are uncommon (Gilbert and Hazelwood, 1993). Xylans constitute the major hemicellulose component in primary cell walls of monocotyledons but occurs in smaller amounts in dicotyledons. Xylans comprise up to 40% of the primary cell wall matrix of gramineous monocots (Aspinall, 1980; Burke *et al.* 1974).

The xylan of hardwoods is *o-acetyl-4-o*-methylglucuronoxylan. This polysaccharide consists of at least 70 β-xylopyranose residues and is highly acetylated. Acetylation occurs more usually at the C-3 than at the C-2 positions, however acetylation at both positions has been reported. On the other hand, softwoods (including cereals) are composed of arabino-4-*o*-methylglucuronoxylans. They have a higher *4-O*-methylglucuronic acid content than do hardwood xylans, and are not acetylated, having instead, α-L-arabinofuranose units linked by α-1,3-glycosidic bonds to the C-3 position to the xylose. The arabinosyl substituents occur on almost 12% of the xylosyl residues (Sunna and Antranikian, 1997). An example of a graminae xylan is shown in Fig 3

iii) Pectin

Two fundamental constituents of pectins are polygalacturonic acid chains (PGAs), which are helical homopolymers of (1→4)α-D-galactosyluronic acid (GalA) and rhamnogalacturonan I (RG I), which are contorted rod-like heteropolymers of repeating (1-2) α-L-rhamnosyl-(1→4) α-D-GalA disaccharide units. The helical chains of PGAs condense by cross-linking with calcium to form junction zones know as multiple egg box structures. Pectin is one of the most complex polymers known and is thought to perform many functions such as determining wall porosity, providing charged surfaces that modulate pH and ion balance, and serving as recognition molecules that signal appropriate developmental responses to symbiotic

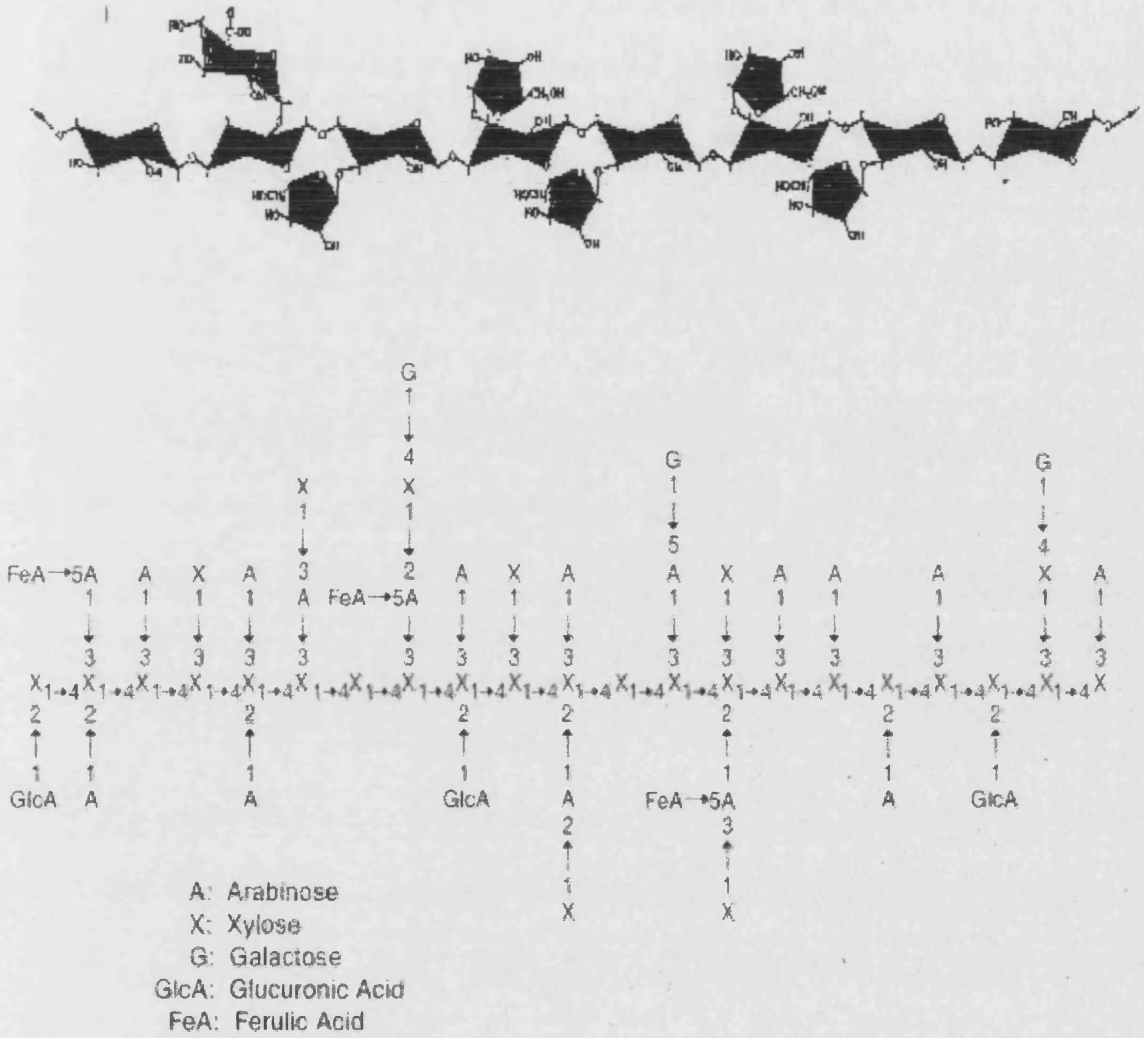


Figure 3 : Typical unit structure of the highly substituted glucuronoarabinoxylans (GAX) of the gramineae cell wall: linear chains of (1→4)β-D-xylose with single arabinose units at the O-3 and less, less frequently, single glucosyluric acid (GlcA) units at the O-2 of the xylosyl units.

organisms, pathogens and insects. Pectins constitute the major matrix polymer in dicots (Carpita and Gibeaut, 1993).

iv) Proteins

Protein is present throughout the cell wall, composing 10-15%, where they serve both structural and enzymatic functions. There are five major classes of cell wall proteins mostly characterized from advanced dicots. These are extensins (hydroxyproline-rich glycoproteins, HRGPs), glycine-rich proteins (GRPs), proline-rich proteins (PRPs), arabino-galactan proteins (AGPs), and solanaceous lectins (Smart, 1991, Showalter, 1993).

HRGPs are found in the primary cell wall acting as structural elements; they can also accumulate in response to penetration attempts by pathogens, surrounding the invading microorganism or acting as a physical barrier or lignin deposition site. The best known of the HRGPs are extensins; which have been correlated positively with resistance and negatively with cell growth. Arabinogalactan proteins (AGPs) like extensins, are rich in hydroxyproline, serine, galactose and arabinose (Hammerschmidt *et al.*, 1984; Fuji *et al.*, 1981). Some of these proteins have been found in different forms in gramineous monocots such as threonine-hydroxyproline-rich glycoproteins (THRGPs) and histidine-rich forms (HHRGPs) (Carlile *et al.*, 2000).

1. 3. 1 Structure of the plant cell wall

Several hypotheses have been proposed to explain how these different polymers are assembled and altered to form an extensible cell wall. In that sense, the more recent models recognise the wall representative of all Dicotyledonae and some Monocotyledonae in one group (type I) and the special wall of the Poeaceae, in the other (type II).

i) Type I cell walls

Fundamentally, Carpita & Gibeaut (1993) consider the Type I primary plant cell wall to be a network of cellulose. The microfibrils are wound around each cell randomly, like threats in a ball of yarn, and this pattern changes during isodiametric expansion. When elongation begins, microfibrils are wound transversely or in a shallow helix around the longitudinal axis.

In this type of wall, the principal interlocking polysaccharides are xyloglucans (Xgs), which comprise linear chains of 1→4 β-D-glucan with numerous xylosyl units added at regular sites at the *O*-6-position of the glucosyl units of the chain. Additional sugars, like β-D-galactose and α-L-arabinose, are added to the *O*-2-position of some xylosyl units. Not all of the Xgs reside as a monolayer coating the microfibrils and much of them expand into the milieu between microfibrils. This cellulose-xyloglucan framework (50% of the wall mass) is embedded in a matrix of pectic polysaccharides (about 30% of the total mass).

An interaction of pectic substances with Xgs may control hydrolysis during cell wall expansion by controlling access of hydrolytic enzymes to the Xgs substrate, since during growth, the orientation of the wall polymers become altered within the matrix. It appears that hydrolytic and transglycosylation reactions in conjunction with dissociation of the linking Xgs allows the helical arranged microfibrils to be pulled apart in the long axis. Once elongation is complete, the primary wall is locked into shape with the action of hydroxyproline-rich glycoprotein extensins (Fig 4).

ii) Type II cell wall

These are composed of cellulose microfibrils similar in structure to those of the type I wall but instead of Xgs, the principal polymers that interlock the microfibrils are glucuronoarabinoxylans (GAXs or Xylans), linear chains of (1→4) β-D-xylose with

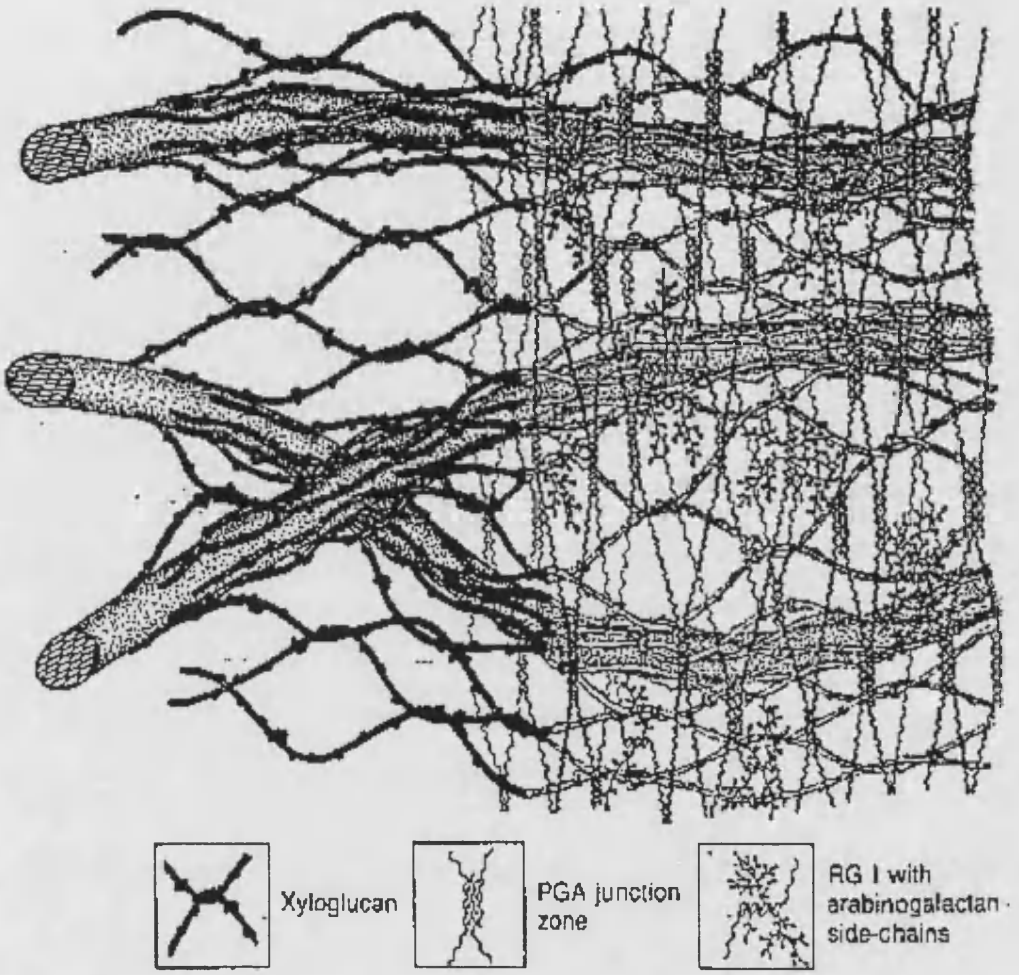


Figure 4 : The type I primary cell wall of most flowering plants (Carpita & Gibeaut, 1993). Representation of a single stratum of the wall just after formation in dividing cells of a meristem. Several strata such as this coalesce to form a wall. The cellulose microfibrils are interlaced with xyloglucan polymers, and this framework is embedded in a matrix of pectin polysaccharides, polygalacturonic acid and rhamnogalacturan, the later substituted with small polymeric side groups of arabinan, galactan, and arabinogalactan. Because xyloglucans have only a single face, which can hydrogen bond to another glucan chain, the xyloglucans are depicted as woven to interlace the microfibrils.

single arabinose units at the *O*-3 and less frequently glucosyluronic acid (Glc A).

The number of side groups of arabinose and Glc A along the xylan chain varies markedly from 10% to 90%. This degree of branching greatly affects the ability of chains to bind to each other. Like Xgs in the type I, the unbranched 1→4 linked xylan can hydrogen bond to cellulose or to each other while the arabinosyl and glucosyluronic groups prevent such bonding, rendering the GAX water-soluble. This characteristic allows GAX to act as an important component of the matrix as pectin does in the type I and accordingly, the spacing of the arabinosyl side units could determine porosity and surface charge (Carpita & Gibeaut, 1993).

Complete breakdown of a branched xylan requires the action of several hydrolytic enzymes, the best known are β-1,4-xylanases (1,4-β-D-xylan xylohydrolases; EC 3.2.1.8) and β-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). In general terms, the xylanases attack internal xylosidic linkages on the backbone and β-xylosidases release xylosyl residues by endwise attack of xylooligosaccharide (Biely, 1985).

However, substituents on xylan main chains are generally considered to interfere with the hydrolysis by these xylanases. The action of accessory enzymes that liberate the side-chain sugars, α-arabinose and α-glucuronic acid (α-arabinofuranoside and α-glucuronidase) may be necessary to achieve its complete breakdown (Nishitani & Nevins, 1990; Ken *et al.* 1988; Biely, 1985).

Most remarkable in the type II wall is the synthesis of polysaccharides specific to cell expansion. The constituents of the wall change markedly when the cells begin to expand. Some arabinans, particularly the 5-linked arabinans, are found in the walls of dividing cells but are no longer made during cell expasion. Instead, mixed linkage (1→3 and 1→4)β-D-glucans are synthesised along with GAX, to fulfil temporarily the interlocking function. When expansion occurs, those glucans are partially hydrolysed and finally disappear when growth is completed. Meaning that unlike XG, β-D-glucans is a stage specific polymer that accumulates only transiently during

expansion and disappears when growth is completed. The fixation of shape then occurs, not through the action of proteins (Hyp-rich extensions) as in type I walls but by esterified and etherified phenolic acids, whose formation is accelerated at the end of growth. Once these phenolic cross-bridges are in place, the load-related role of β-D-glucan is terminated, and the polymer can be hydrolysed completely with negligible impact on wall physics. Fig 5 shows the model of final stage of the type II wall (Carpita & Gibeaut, 1993).

1. 3 Role of CWDE as Pathogenicity Factors

The plant cell wall constitutes a potential barrier to invasion and a nutritional source for pathogenic and saprophytic microorganisms and in concordance, extracellular cell wall-degrading enzymes are produced by most of them. De Bary (1886) was the first to suggest that extracellular enzymes may be involved in the infection process of plant pathogenic fungi. Since then, many such enzymes from a number of pathogens have been studied for a possible role in various aspects of pathogenesis, including penetration, nutrient assimilation, tissue colonisation, symptom expression and elicitation of host defence responses (Cooper, 1983; Van Hoof *et al.* 1991).

Traditionally, this has been done by studying their production *in vitro* on host cell walls and when grown in infected tissues, after that purifying and characterising CWDE and examining effects of purified or partially purified enzymes on plant cells. (Cooper and Wood, 1975; Cooper, 1983). Using these approaches it has been demonstrated that pathogens can adapt their CWDE depending on the nature of the host cell wall (Cooper, 1984; Cooper *et al.*, 1988). Pathogens of cereals have as main activities xylanase and α-arabinosidase and subsequently β-1,3-glucanase, corresponding to the predominant cell wall matrix polymer (xylan), and mixed glucans and possibly callose produced in response to parasite penetration. Pectic enzyme levels remain low and appear late in contrast to pathogens of dicots; however, research concerning the effect of pathogen CWDE in during penetration of grass

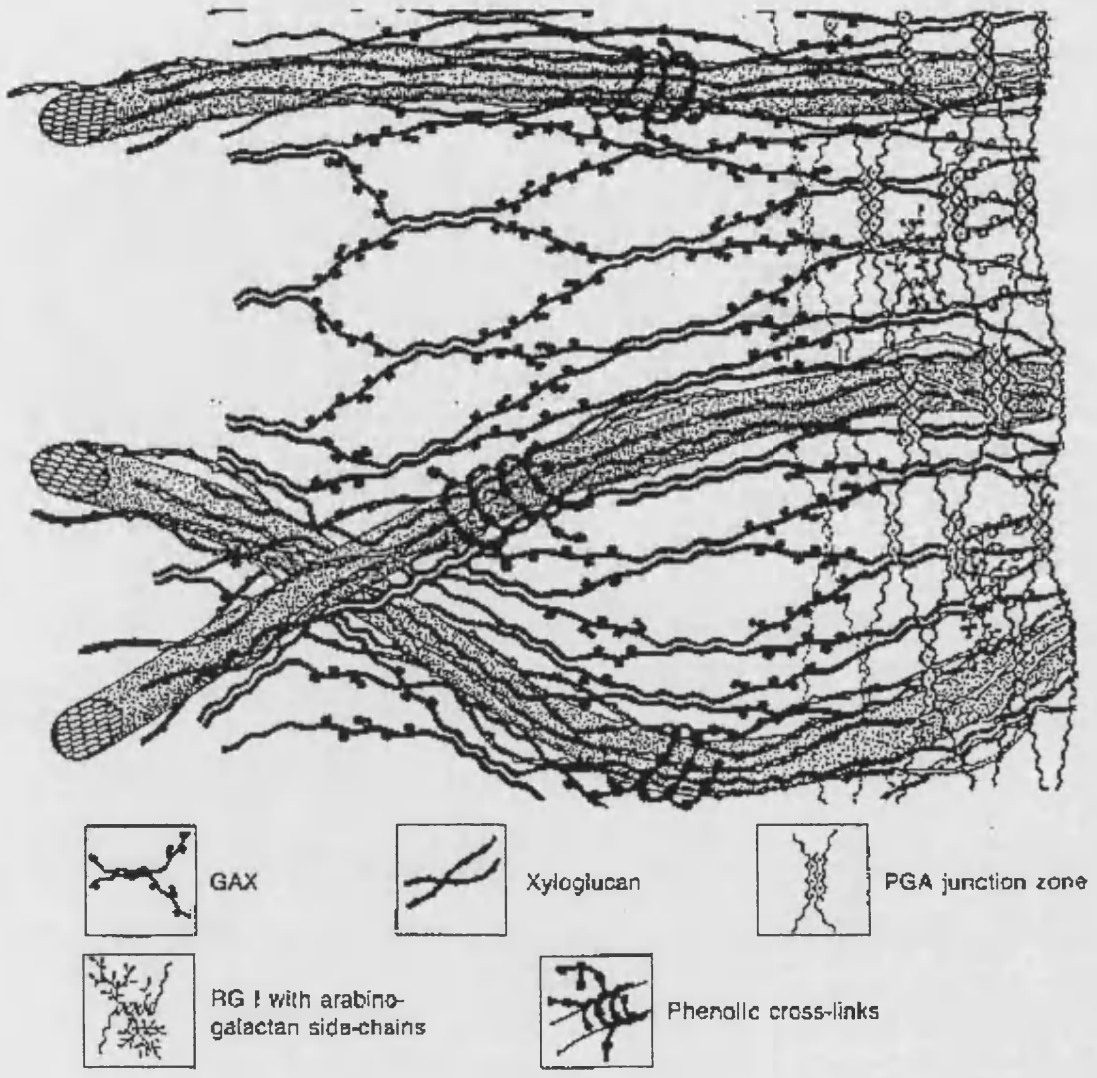


Figure 5 : The type II cell wall of the Poaceae (Carpita & Gibeaut, 1993). Representation of a single stratum of the wall just after cell division. The microfibrils are interlocked by glucuronoarabinoxylans (GAX) instead of xyloglucans. Unlike xyloglucans, the xylans are substituted with arabinosyl units, which block hydrogen bonding. Porosity of the GAX domain could be determined by the extend of removal of the appendant units. Some highly substituted GAX remains intercalated in the small amount of pectins that are also found in the primary wall. Unlike the type I wall, substantial portions of the non-cellulosic polymers are "wired on" the microfibrils by alkali-resistant phenolic linkages.

tissues by fungi or the significance of these enzymes in pathogenesis is still scarce (Cooper *et al.*, 1988; Holden & Walton,1992; Peltonen *et al.*, 1994; Wu *et al.*, 1995).

The evidence accumulated suggests that CWDE may play a determinant role in pathogenesis. Conversely, Lotan and Fluhr (1990) demonstrated that an endo-1,4-β-xylanase purified from culture filtrate of *Trichoderma viride* can elicit the accumulation of pathogenesis related proteins (PRP) when applied directly to tobacco leaves. Also, it has been shown that CWDE may be particularly important for those pathogens that invade their host by penetrating through intact surfaces (Walton 1994; Lehtinen, 1993; Keon *et al.*, 1987).

Despite considerable progress, definitive conclusions on the importance of CWDE to plant pathogenic fungi have not been reached. However, in recent years, recombinant DNA techniques have been employed to try to provide more conclusive evidence concerning the role of CWDE in plant pathogenesis. Many groups have taken a direct approach to clone and disrupt or delete genes whose products were suspected to be required for disease development. Such genes include those encoding cell wall-degrading enzymes and enzymes degrading preformed or induced anti-fungal toxins and those involved in phytotoxin synthesis (Oliver and Osbourn, 1995). Unfortunately, the outcome of most attempts with transformants for disrupted CWDE has not given indication of their requirement for patogenicity.

Others have tried with the identification and isolation of pathogen genes expressed specifically during infection of the host plant. These experiments have provided an alternative to predictive approaches for the identification of pathogenicity determinants. Differential screening of *Phytophthora infestans* genomic DNA library with cDNA from infected plants and from *in vitro*-grown fungus identified nine genes that exhibited *in planta*-induced expression (Pieterse *et al.*, 1994a, 1994b). The identity of many of these genes and their requirement for pathogenicity are still unclear. The *MPGI* gene of *Magnaporthe grisea* was isolated by the differential screening of a cDNA library constructed from *M. grisea*-infected rice leaves.

Presumed to encode a hydrophobin, its disruption resulted in a significant reduction of virulence (Talbot *et al.*, 1993)

1. 3. 1. Cutinases

The aerial parts of plants are protected by the cuticle which is composed of an insoluble polyester, called cutin, embedded in a complex mixture of nonpolar lipids collectively called waxes. This polymer constitutes one of the major physical barriers to penetration of fungi into the aerial parts of plants. Cutinase and its role in the infection process have been studied intensively in *Fusarium solani* f.sp *pisi*. Cutinase inhibitors such as anticutinase IgG or chemical inhibitors such as organophosphates were able to prevent lesion formation, solely by preventing penetration rather than by being toxic to the fungus (Kolattukudy and Crawford, 1987).

The insertion of the gene encoding this cutinase into the wound pathogen of papaya fruits, a *Mycosphaerella sp.* enabled the fungus to infect the host through the intact cuticle (Dickman *et al.*, 1989). Similarly, *Alternaria alternata* mutants deficient in cutinase showed reduced pathogenicity towards pear and cutinase inhibitors also prevented penetration (Tanabe *et al.*, 1988a,b).

In contrast, Stahl and Schafer (1992) created a cutinase-deficient mutant in *Fusarium solani* f.sp. *pisi* by transformation-mediated gene disruption. The mutant showed no reduction in pathogenicity and virulence, contradicting the requirement of this activity in pathogenicity. Disruption of the cutinase gene of *M. grisea* failed to alter pathogenicity, however residual activity remained and there is strong evidence for mechanical penetration (Sweigard *et al.*, 1992).

1. 3. 2 Pectinases

Pectic polysaccharide have been the most studied CWDE. The enzymes capable of attacking the pectic fraction of plant cell walls are the only ones that have become generally accepted as pathogenicity factors. They are produced in multiple isoforms of

different kinds of pectolytic enzymes. Pectinases are typically produced first *in planta* and in culture on host cell walls, and may be the dominant activities; they are the only CWDE capable of macerating plant tissue and killing plant cells independently (Collmer and Keen, 1986; Cooper, 1984; Cooper, 1983).

Pectins are the most complex plant cell wall polysaccharides due to the numerous sugar monomers and types of linkages involved in the branched rhamnogalacturans I and II domains, and to the variable level of esterification of the homogalacturonan domain. Pectins interact with diverse ions most notably calcium and boron, and as a polyanion has the potential to interact with polycations. These structural features confer important roles to pectic polysaccharides, one of them being to cement plant cells (Esquerre-Tugaye *et al.*, 2000).

Pectinases, most notably polygalacturonases, are classified by their substrates, type of lysis and mode of action on the pectin polymer. Unesterified pectate polymers can be degraded by polygalacturonase (PG) that uses hydrolytic cleavage, and pectate lyase (PL), which uses β-elimination cleavage. Esterified pectin polymers are attacked by pectin lyase (PNL) or polymethylgalacturonase. Rhamnogalacturonase (RHG) cleaves the bond between the alternating galacturonic acid and rhamnose residues in rhamnogalacturan. Two types of pectinases have been differentiated by their cleavage pattern: an endo form that cleaves internal regions of pectin chains randomly and exo form that removes terminal residues (Esquerre-Tugaye *et al.*, 2000; Annis and Goodwin, 1997; Cooper, 1983).

Pectin methyl esterase (PME), which is also produced by fungi and plants, removes the methyl group from esterified galacturonic acid residues in pectin chains and thereby decreases the steric hinderance to other pectinaes allowing them to attack the backbone of the pectin chain. For each type of pectinase, as well as many other CWDE, most fungi appear to produce multiple isoenzymes that differ in isoelectric point, molecular weight and often in their regulation. This multiplicity may give flexibility to a pathogen, with each enzyme having its own unique properties that contribute to the performance of all the enzymes (Keon *et al.*, 1987).

Initial attack by pectic enzymes is known as a prerequisite for the activity of other CWDEs, including cellulases. In other words, a pathogen may not successfully attack the host unless enough polygalacturonase is produced to overcome the inhibitors existing or produced by the plant in response to the pathogen's attack (Deising *et al.*, 1992).

However it has been proposed that penetration of intact cell walls can occur without pectic enzyme activity in pathogens of graminaceous plants, such as *Rhizoctonia cerealis* and *Pseudocercosporella herpotrichoides*, where these enzymes are only detected late in infection and at a low levels, which might be because of the relatively small amount of pectic polymers in monocot cell walls (Keon *et al.*, 1987).

The role of pectic enzymes in tissue maceration has been studied in genetic transformation experiments in which the non-phytopathogenic *E. coli* was transformed with the genes encoding the pectic enzymes from the phytopathogen *Erwinia chrysanthemi* (Zink and Chatterjee, 1985). This resulted in some endo-pectate lyase producing transformants capable of macerating and killing plant tissues, although, very large inoculum was required to initiate colonisation of tissues by these transformants, suggesting that secretion of pectic enzymes alone is insufficient to ensure plant parasitism. On the other hand, individual lyases of *Erwinia* spp have been disrupted alone and in combinations; some resulted essential to pathogenicity others did not and one affected systemic spread (Boccara *et al.*, 1988).

Employing disruption of double (endo and exo) or single polygalacturonase genes, Scott-Craig *et al.* (1990, 1998) and Gao *et al.* (1996) did not observe any significant redution in pathogenicity of *Cochliobolus carbonum* and the chestnut pathogen *Cryphonectria parasitica* towards maize and chestnut, respectively, in the mutants. In both cases, a low level of polygalacturonase activity was still detectable in the mutants, thus the possibility that polygalacturonase is important for plant invasion cannot be ruled out.

1. 3. 3 Xylanolytic enzyme system

In contrast to the dicot wall where pectin is the main matrix polymer, in graminaceous monocots this is largely replaced by xylan. Xylan is the major interlocking polymer and constitutes up to 40% of the wall. Its degradation could considerably weaken the wall and allow access for cellulases to their substrate The circumstantial evidence for xylanases in disease is not as extensive as for pectinases, particularly because of their inability to cause tissue maceration directly. Despite this, xylanases have been demonstrated to be capable of degrading isolated cell walls (Carmona *et al.*, 1998; Baker *et al.*, 1977; Ramson and Walton, 1997).

Xylans are structurally of mixed composition and thus the enzymatic degradation of the substrate to its monomer xylose is a complex process involving a battery of enzymes (He *et al.*, 1993; Biely, 1985). This is usually composed of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase and phenolic acid esterases. All these enzymes act co-operatively to convert xylan to its constituent components (Fig 6). The presence of such multifunctional xylanolytic enzyme systems is quite widespread among bacteria and fungi (Filho *et al.*, 1996; Coughlan *et al.*, 1993; Wong *et al.*, 1988).

Endoxylanase and β-xylosidase have the most important activities among the xylanolytic enzymes involved in xylan hydrolysis. Side chains cleaving enzymes like α-arabinofuranosidase, α-glucuronidase, and acetylxylan esterase play important roles in the removal of side substituents of heteroxylans. Substituent groups in xylan are a limiting factor in achieving the efficient hydrolysis of the substrate (Kubata *et al.*, 1994; Debeire *et al.*, 1990).

1. 3. 3. 1. Endoxylanase

β-1,4-Endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased average

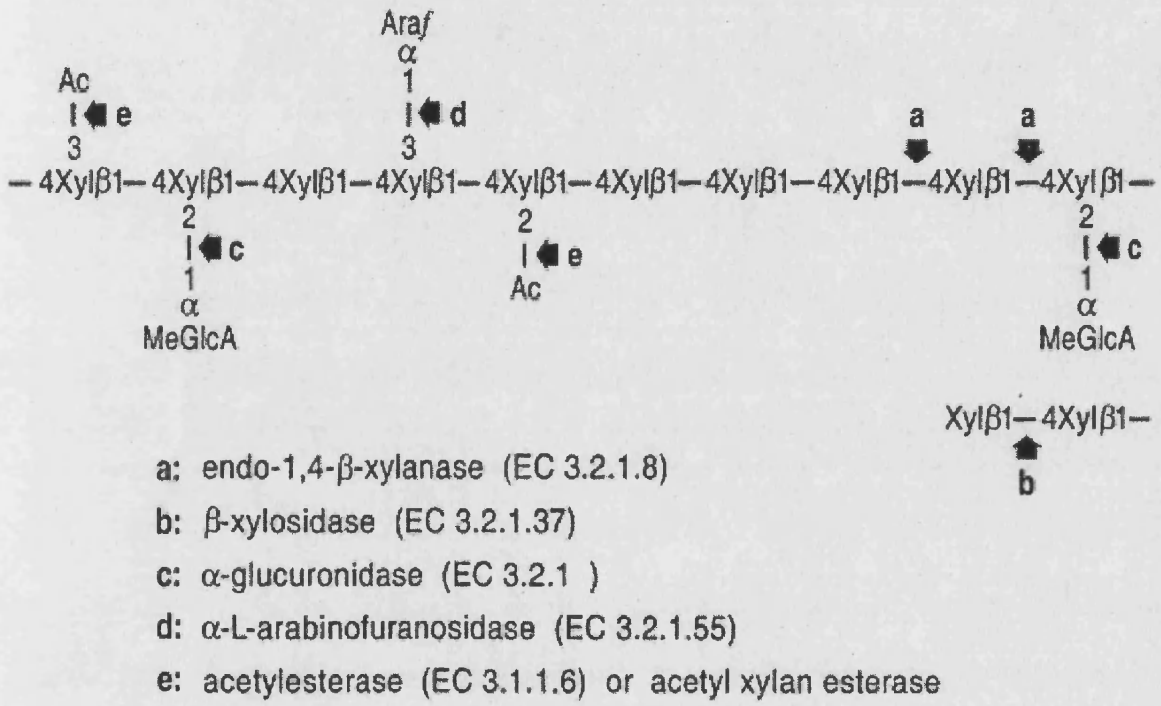


Figure 6 : The xylanolytic enzymes involved in the degradation of xylan. Ac; acetyl group; α-Ara*f*: arabinofuranosyl group; α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid; Xylβ1-4 Xylβ1- xylobiose or xylooligosaccharide.

degree of polymerisation (DP) of the substrate. The attack of the substrate is not random, and the bonds to be hydrolysed depend on the nature of the substrate (e.g. length and degree of branching of the substrate or the presence of substituents). During the early course of hydrolysis of xylan the main products formed are xylo-oligosaccharides that are further hydrolysed to xylotriose, xylobiose and xylose. Endo-acting xylanases have been differentiated according to the end products released from the hydrolysis of xylan: nondebranching (arabinose non-liberating) or debranching (arabinose-liberating) enzymes. Many organisms are able to produce both types of enzymes, resulting in the maximum efficiency of xylan hydrolysis (Debyser *et al.*, 1997; De segura *et al.*, 1993; Wong *et al.*, 1988).

Wong *et al.* (1988) have suggested that endoxylanases could be grouped into those that are basic proteins with MW below 30,000 and those that are acidic with MW above 30,000; though, several exceptions to this general pattern have been reported.

The optimum temperature for endoxylanases from bacterial and fungal sources varies between 40 and 60 °C. Fungal xylanases are generally less thermostable than bacterial xylanases. Xylanases from different organisms are usually stable over a wide pH range (3-10) and show optimum pH in the range to 4-7. The isoelectric points for endoxylanases from various sources ranged from 3 to 10 (Biely, 1993).

Xylanases can be grouped into families on the basis of conserved amino acid sequences on the catalytic domains and by hydrophobic cluster analysis. Thus, all high molecular-weight xylanases belong to the F family of glycanases, whereas low-molecular weight xylanases belong to the G family (Henrissat, 1992; Gilkes *et al.*, 1991). Based on their physicochemical properties, they can be divided into low-molecular-weight/basic (group 1) and high molecular-weight/acidic proteins (group 2). However there are several exceptions to this general pattern and endoxylanases with low pI and low MW values have been reported, and vice versa.

Xylanase production by various bacteria and fungi has been shown to be inducible (Biely *et al.*, 1980; Gaspar *e t al.*, 1997; Mohand-Oussaid *et al.*, 1999;

Tonukari *et al.*, 2000). But examples of constitutive xylanase expression have also been reported (Srivastava and Srivastava, 1993). In general the xylanase regulation is a complex phenomenon and the level of response to an individual inducer varies with the organisms. The substrate derivatives and the enzymatic end products may often play a key role in the induction of xylanases; they can also act as the end product inhibitors, possibly at much higher concentrations. A hypothetical model of the regulation of xylanase biosynthesis is diagrammatically represented (Fig 7), based on the figure by Thomson (1993).

1. 3. 3. 2 β-Xylosidase

β-D-xylosidases (β-D-xylosidase xylanohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyse short xylooligosaccharides and xylobiose from nonreducing end to liberate xylose (Wong *et al.*, 1988). True β-xylosidases are able to cleave *p*-nitrophenyl-β-D-xyloside. These enzymes have been reported in fungi and bacteria, with molecular weights between 60 and 360 kDa, and are mono- or dimeric proteins (Coughlan and Hazlewood, 1993).

β-Xylosidases appear to be mainly cell associated in bacteria and yeast. However extracellular β-xylosidase activity has also been reported. In the yeast *Cryptococcus albidus*, xylobiose and xylotriose enter the cells through a β-xyloside permease transport system and are converted by β-xylosidases to xylose (Biely *et al.*, 1980).

Generally, purified β-xylosidases are unable to hydrolyse xylan. However, there are some reports of β-xylosidases that are able to attack xylan slowly to produce xylose. Among the xylooligomers, xylobiose is usually the best substrate (Ramson and Walton 1997).

A number of β-xylosidases has been reported to possess α-arabinosidase activity. Among them enzymes from *A. niger* (Radinova *et al.*, 1983); *T. reesei* (Poutanen and Puls, 1988) and *T. ethanolicus* (Shao and Wiegel, 1992). Most of these reported β-

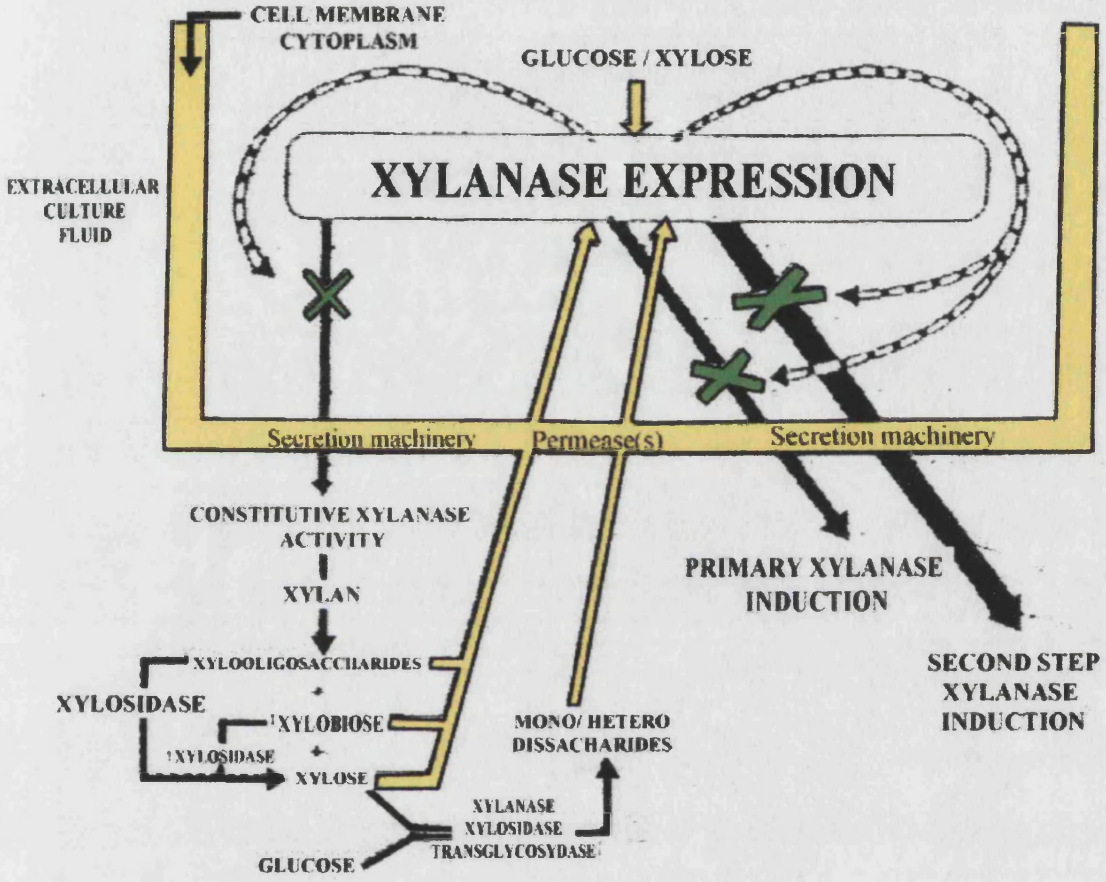


Figure 7 : Regulation of xylanase biosynthesis (hypotetical model). Constitutive xylanases degrade xylan to xylooligosaccharides and xylobiose, which are taken up by the cell and induce other xylanase genes. The inducible xylanases degrade xylan further to xylooligosachharides and xylobiose. The β-xylosidases, which may be produced constitutively and /or inducibly, conver xylobiose to xylose and subsequently transglycosilate it to XylB1-2Xyl and GlcB1-2Xyl. These compounds are taken up by the cell and act as additional inducers of genes encoding xylanolytic enzymes (based on Thomson, 1993)

xylosidases are inhibited by their hydrolysis product xylose. An important role of β-xylosidases seems to be relieving the end product inhibition of endoxylanase (Thomson 1993).

1. 3. 3. 3 α-L-Arabinofuranosidase

Enzymes hydrolysing L-arabinose linkages have been purified from several bacteria and fungi. They can be classified according to the substrate molecular weight, *i.e.*, those that are specific for L-arabinosides with low molecular weights such as synthetic substrates (*p*-nitrophenyl-α-L-arabinofuranoside) and L-arabinooligosaccharides are named α-L-arabinofuranosidase (EC 3.2.1.55); secondly, those named endo-1,5-α-L-arabinase (EC 3.2.1.99), specific for L-arabinans., but are not able to hydrolyse *p*-nitrophenyl-α-L-arabinofuranoside or gum Arabic (Fernandez-Espinar *et al.*, 1994; Filho *et al.*, 1996).

α-L-Arabinofuranosidases capable of hydrolysing both 1,3- and 1,5-α-L-arabinofuranosyl linkages in arabinoxylan have been reported. Equally, an arabinosidase has been purified that is highly specific for arabinoxylans (1,4-β-D-arabinoxylan arabinofuranosidase), and is able to release only arabinose from arabinoxylan, while the xylan backbone is not degraded, and no production of xylooligosaccharides is made during its activity (Kormelink *et al.*, 1991; Nishitani and Nevins, 1991).

Most of the arabinan-degrading enzymes studied so far are of the exo-acting type. The size of the native arabinofuranosidases may reach up to 495 kDa and are found in mono-, di-, tetra-, hexa-, and octameric forms. The production of this enzyme seems to be induced, among others, by arabinan, xylan, and wheat bran. On the other hand, only low levels of this enzyme have been detected when the organisms were grown with glucose or cellobiose as a substrate (Luonteri *et al.*, 1995).

There is little evidence suggesting that arabinofuranosidases are required for

pathogenesis. Arabinases and arabinosidases are among the first enzymes produced when several pathogens of monocotyledonous plants are grown on isolated monocotyledonous cell walls (Cooper *et al.*, 1988, Ramson and Walton, 1997). Extracellular α-arabinosidase has been purified from the plant pathogenic fungus *Sclerotinia sclerotium* (Baker *et al.*, 1979). Genetically uncharacterised mutants of *S. fructigena* and *S. trifoliorum* that are deficient in α-arabinosidase activity have shown reduced virulence (Rehnstrom *et al.*, 1994).

Synergism between α-L-arabinofuranosidase and xylanases has been reported. An increase in xylose, xylobiose and arabinose production occurred when both enzymes were used simultaneously (Greve *et al.*, 1984; Poutanen, 1988).

Several genes encoding a-L- arabinofuranosidases have been isolated from bacteria, such as *Butyrivibrio fibrisolvens*, *Streptomyces lividans*, *Pseudomonas* sp, among many others. However, few genes have been isolated from fungal species, mainly from *Aspergillus* sp and *Trichoderma* sp (Flipphi et al., 1993; Margolles-Clark *et al.*, 1996), but apparently not from any plant pathogenic fungi (Ramsom and Walton, 1997).

1. 3. 3. 4 α-Glucuronidases

α-D-Glucuronidases (EC 3.2.1.) hydrolyse the α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. Due to the lack of glucuronidase activity in many fungal hemicellulase preparations (Puls and Poutanen, 1989), this enzyme was not described until 1986 (Puls *et al.*, 1986). Despite the role played by α-glucuronidases in the biodegradation of xylan, few enzymes have been reported so far; most of them have molecular weights around 100 kDa. (Sunna and Antranikian, 1998). The substrate specificities of α-glucuronidases differ according to the enzyme source. The enzymes from *Agaricus bisporus* and *Streptomyces olivochromogens* require a low-molecular weight glucuronoxylan substrate. They release 4-*O*-methylglucuronic acid from 4-*O*-methyl-glucuronose-substituted xylooligomers, but

not from the polymer (Puls *et al.*, 1987; Johnson *et al.*, 1989). On the other hand, the α-glucuronidases produced by *Schizophyllum commune* are able to liberate 4-*O*-methylglucuronic acid from methylglucuronoxylan (Johnson *et al.*, 1989).

1. 3. 3. 5 Acetylxylan esterases

Acetylxylan esterases (EC 3.1.1.6) remove the *O*-acetyl substituents at the C-2 and C-3 positions of xylose residues in acetylxylan. The production of acetylxylan esterases by fungi and bacteria was reported relatively recently due to lack of suitable substrates (Biely, 1985). Although xylan is highly acetylated in its native state, most of the xylans used to study xylanolytic enzyme systems were deacetylated xylans obtained from alkali extractions. Few acetylxylan esterases have been purified and characterised and little is known about their physicochemical properties (Tenkanen and Poutanen, 1992).

An example of a described acetylxylan esterases is that of *Thermoanaerobacterium* sp., which is composed of acetyl esterase II and I. Acetyl esterase II removes the acetyl groups from xylan extracellularly, whereas acetylxylan esterase I acts on short acetylated xylooligosaccharides intracellularly (Shao and Wiegel, 1995).

Acetyl groups present on the xylan backbone inhibit the action of xylanases by steric hindrance. Acetylxylan esterases may play an important role in relieving this inhibition, therefore by forming new unsubstituted sites on the polysaccharide backbone. This therefore increases the susceptibility of the polymeric xylan to endoxylanase (Biely, 1985).

1. 3. 3. 6 Ferulic and *p*-Coumaric acid esterases

Esterified hydroxycinnamic acids, notably ferulic and *p*-coumaric acids, occur widely in the cell wall of plants. In graminaceous plants, hydroxycinnamic acids are mainly

found linked to xylans by ester bounds to the *O*-5 position of the arabinose substituents in arabinoxylan. These compounds contribute to the structural integrity of the cell wall by cross-linking between hemicellulose chains and between ligning and hemicellulose. Ferulic acid esterases (EC 3.1.1.) cleave the ester linkages between arabinose side chains and ferulic acids in xylan. Similarly, *p*-coumaric acid esterase (EC 3.1.) cleaves the ester linkage between arabinose and *p*-coumaric acid (Fillingham *et al.*, 1999).

Few of these enzymes have been purified and characterised. Among them are those of the fungus *Neocallimastrix* strain MC-2. This anaerobic fungus produces a *p*-coumaroyl and two feruloyl esterases. The purified *p*-coumaroyl esterase is a dimer with MW of 11 kDa and is neither able to release ester-linked acetyl groups from xylan, nor reducing sugars from oat spelt xylan; the two feruloyl esterases are monomeric, with MW of 68 and 24 kDa. These enzymes release ferulic acid when incubated with ferulic acid linked to tri- and tetra saccharides (Borneman *et al.*, 1993).

1. 3. 3. .7 Synergism Between Xylan-Degrading Enzymes.

According to the reports reviewed, it seems that xylan hydrolysis requires the synergistic action of enzymes acting on the 1,4-β-D-xylan backbone and side chain cleaving enzymes. Multienzyme complexes analogous to cellulosomes and capable of efficient degradation of xylan can be found on the surface of several microorganisms.

Some remarkable examples are the multicomplex hemicellulose systems of *Butyrivibrio fibrisolvens* (Lin and Thomson, 1991); *Clostridium papyrosolvens* (Pohlschroder *et al.*, 1994); *Clostridium cellulolyticum* (Mohand-Oussaid *et al.*, 1999); *Penicillium canescens* (Gaspar *et al.*, 1997) and the anaerobic fungus *Piromyces* sp. (Ali *et al.*, 1995 and Fillingham *et al.*, 1999). All of them possess several protein bands with xylanase, glycosidases and esterases activities, added to the presence of a noncatalytic substrate binding glycoprotein, which serves as a carrier

keeping all enzymes acting together; or binding each other through cellulose-binding domains in the polypeptide chains.

In fact, the cooperative action between xylanases and debranching enzymes has been demonstrated. For example, the synergistic action between acetylxylan esterase and endoxylanases results in the efficient degradation of acetylated xylan. The release of acetic acid by acetylxylan esterase increases the accessibility of the polysaccharide backbone for endoxylanase attack. The endoxylanase creates shorter acetylated polymers, which are the preferred substrates for esterase activity (Biely, 1985; Biely *et al.*, 1988).

Synergism between the xylanases and α-arabinofuranosidases was described for the enzyme systems of *Talaromyces emersonii.* The xylanases II and III from *T. emersonii* were unable to hydrolyse wheat straw arabinoxylan unless the polymer had been previously treated with an α-arabinofuranosidase of the same organism (Tuohy *et al.*, 1993).

The thermophilic actinomycete *Thermomonospora fusca* possesses a complex, multicomponent, xylan-degrading enzyme system that consists of endoxylanase, β-xylosidase, α-arabinofuranosidase and acetyl esterase activities (Bachmann and McCarty, 1991). Similarly, the synergistic effects among endo-xylanase, β-xylosidase and α-L-arabinofuranosidase from *Bacillus stearothermophilus* was demonstrated (Suh *et al.*, 1996) The hydrolysis of xylan by endoxylanase was enhanced by the addition of β-xylosidase, which seems to be responsible for relieving the endo-product inhibition of endoxylanase. Similarly, the addition of α-arabinofuranosidase to endoxylanase enhanced the saccharification of arabinoxylan. The incubation of oat spelt xylan with endoxylanase and β-xylosidase from *Bacillus stearothermophilus* caused the complete conversion of this substrate to xylose (Nanmori *et al.*, 1990). Furthermore, there is evidence that removal of arabinose substituents is a requisite for the endoxylanase to have access to the xylan backbone in *Fibrobacter succinogens* (Matte and Forsberg, 1992).

Synergism between phenolic acid esterases and xylanases has been reported. The amount of released *p*-coumaroyl and feruloyl groups from coastal Bermuda grass increased when the *p*-coumaroyl and feruloyl esterases from *Neocallimastrix* strain MC-2 were incubated with xylanase. The polymer-degrading enzyme may provide free phenolic oligosaccharides, which act as a substrate for the phenolic acid esterases (Bonerman *et al.*, 1993).

Synergism also occurs between various endoxylanases. For example, *Fibrobacter succinogenes* S85 secretes two types of endoxylanases; unlike endoxylanase 2, endoxylanase 1 possesses a debranching activity (arabinoxylanase), liberating arabinose and xylooligomers from xylan. The release of arabinose precedes the complete hydrolysis of xylooligomers by endoxylanase 2 (Matte and Forsberg, 1992).

Considering the complex nature of xylan, its important role in the architecture of the cell wall of the Poaceae and the possibility that enzymes degrading xylan may be key factors in the pathogenicity of a fungal pathogen towards a graminaceous plant, the aims of this study were as follows: To assess the potential range of CWDE produced by *Stagonospora nodorum* growing on wheat cell walls and particularly those present in early stages of infection after inoculation of wheat leaves. Then to select those enzymes that seemed to be implicated in pathogenicity for further purification and biochemical study. Finally, cloning and sequencing of these genes and genetic analysis would reveal any similarities with other microbial depolymerases and enable their potential for targeted gene disruption experiments in order to assess critically the role of these CWDE in pathogenesis.

CHAPTER 2 PRODUCTION OF CELL WALL DEGRADING ENZYMES (CWDE) BY *Stagonospora nodorum IN VITRO* AND *IN PLANTA.*

2. 1 Introduction

The degradation of structural polymers of plant cell walls might be significant during penetration and colonisation of plants by microbial plant pathogens. This is because plant pathogens must encounter and deal with the cell wall that exists as a potential barrier to invasion. According to how this barrier is overcome, the pathogen might make use of the cell wall polymers as a carbon source by degrading them to use monomers or dimers while avoiding formation of signalling molecules that would trigger prompt host defence responses. This process could then be a critical determinant in a compatible plant-microbe interaction. Although the involvement of wall-degrading enzymes and their genes in penetration, pathogen ramification, plant defence induction, and symptom expression has been studied extensively, conclusive evidence for or against a role for any particular enzyme activity in any aspect of pathogenesis has been difficult to obtain (Walton 1994).

According to Cooper (1983), to assess the significance of microbial cell wall degrading enzymes in pathogenesis, several criteria should be fulfilled. Among them are the ability to produce CWDE *in vitro*, detection of CWDE in infected tissues, correlation of enzyme production with pathogenicity and reproduction of wall changes or disease symptoms with purified enzymes.

Detection of polysaccharidases of pathogens grown on isolated plant cell walls *in vitro* and a comparison of levels and types of activity with those in infected tissues can be used as an indication of the influence of CWDE during the process of pathogenicity. In

addition, an analysis of the detected isoforms concomitant with total activities *in vivo* and *in vitro*, should give a clearer picture of the pathogen's CWDE and their possible involvement in pathogenicity and the development of symptoms.

Detached seedling leaves have been used in the rapid screening of cereal breeding lines for their reaction to *S. nodorum*. Positive correlations have been established between the symptoms produced on leaves mounted on agar and the symptoms produced in plant leaves in the field (Caten and Newton, 2000; Newton and Caten, 1988; Benedikz *et al.*, 1981).

Liquid cultures with wheat cell walls as a single source of carbon as well as inoculated leaves have been used in the study of *S. nodorum* disease of wheat for detection of enzyme production and isoforms (Lehtinen, 1993; Magro, 1984). The results showed that a range of CWDE was produced reflecting the composition of the wheat cell wall. Xylanase activity predominated but the number of xylanase isoforms between the two studies was not in agreement. There is evidence of direct penetration and subsequent intercellular colonisation with the apparent involvement of enzyme action on the cell wall, as observed by transmission electron microscopy (Karjalainen & Lounatmaa, 1986). Also, a previous study carried out within our group (Carlile *et al.*, 2000) had detected high levels of xylanase by *S nodorum in vitro* and in inoculated plants, but failed to determine the isoforms of this enzyme.

Here, inoculation of detached seedling leaves as an *in planta* system and liquid culture with wheat cell wall as a single carbon source as an *in vitro* system, were used with the aim of determining the potential range of CWDE produced by *S nodorum* and which enzymes appear to be implicated in the pathogenicity towards wheat. This would be attempted by comparison of kinetics of their expression in both systems, and by identification of the corresponding fungal isoenzymes.

2. 2 Materials and Methods

2. 2. 1 Isolation, Growth and Maintenance of *S. nodorum*

Stagonospora nodorum isolate BS171, adapted to wheat (Osbourn *et al.*, 1986) was obtained from AgrEvo UK Ltd. and isolate BS471, Ln 97 and LAW adapted to wheat, were obtained from Dr. C.E. Caten, School of Biological Sciences, University of Birmingham.

2. 2. 1. 1 Passage Through Wheat and Monospore Isolation

In order to keep the virulence of the strains and also to keep strain 471 producing spores (since it lost the ability to sporulate after three subcultures on agar plates) all the strains were passaged through wheat. After inoculating some susceptible wheat plants by brushing the surface of the leaves with spore suspensions, single infected leaves with each strain were removed. Segments bearing a single defined lesion were excised, washed in 0.02% (v/v) Tween 80 (BDH) in water and brought to aseptic conditions. Leaf segments were surface sterilised by immersion in 70% (v/v) ethanol for 1 min followed by another immersion in 0.5% sodium hypochlorite for 5 min and three washes in sterile distilled water for 5 min each. The surface sterilised segments were then placed in Petri dishes containing media composed of 0.5 % (w/v) technical agar (Oxoid No3), 0.5 % (w/v) wheat cell wall (see 2.2.2.1), in basal salts solution (see 2.2.1.2). These plates were incubated at 20°C until hyphae developed and then, master cultures were selected by choosing a strain from each lesion, which were plated again and kept at 4°C.

From master cultures, monospore isolation was carried out by placing segments of hyphae on Czapek-Dox-V_8 agar plus complete supplement (appendix 1). These plates

were incubated at 20 °C under continuous UV light for 7 days to induce spore production. Spores were visible as a pink mass (cirrhus) oozing from the pycnidia.

A single cirrhus was chosen from each strain and with a fine sterile needle, transferred into 1 ml of sterile distilled water and mixed thoroughly to obtain spore suspensions. Dilution of these suspensions were plated on Czapek-Dox-CS-V_8 agar media and incubated to give discrete single-spore colonies. A single-spore colony was selected for each isolate.

Spore stocks were prepared by flooding single-spore colony plates with 0.02% Tween 20 (BDH) in sterile distilled water and scraping the surface to release conidia. This spore suspension was filtered through muslin to remove mycelium before centrifugation at 10.000xg for 10 min. Spores were resuspended in an equal volume of fresh water and then centrifuged again at 10.000xg for 10 min, to remove any germination inhibitors. The washed spores were plated again until a good number of spores were obtained. For long term storage, the spores were transferred into 20 %(v/v) glycerol at $2x10^7$ spores / ml, and frozen as 1 ml aliquots at -20 °C. Subcultures were made no more than twice, then reisolation was from master cultures.

2. 2. 1. 2 Liquid Culture

Established biomass cultures were produced by inoculating 20 ml of CzV_8Cs liquid medium (appendix 1.1) with $2x10^6$ spores and growing at 25 °C at 125 rpm for 5 days. Mycelium was washed with 50 mM MES pH 6.0 and then transferred to a basal salts liquid medium for 12 h of starvation. Basal salts were composed of (g/l): 1g KH_2PO_4, 2g $NaNO_3$, 0.5g $MgSO_4.7H_2O$, 9.76g MES (50 mM) and 10 ml trace element solution (appendix 2).

2. 2. 2 Growth and Inoculation of Wheat Plants

Wheat plants variety Riband were supplied by PBI Cambridge. Plants were grown at 18±2°C 12 h light /dark cycle for 14 days, when one leaf was fully extended for inoculation or for preparation of cell wall. Plants were grown in trays of 35x24 cm in which seeds were evenly distributed.

2. 2. 2. 1 Preparation of Wheat Cell Walls

Cell wall material was prepared by grinding chopped leaves in liquid nitrogen with a pestle and mortar. Ground plant material was suspended in 2.5 volumes of cold 0.1 M phosphate buffer pH 6 before comminution in a Waring Blender on high speed for 3 min. The resulting suspension was filtered through 2 layers of muslin and the insoluble plant material resuspended in two volumes of cold phosphate buffer and allowed to stand for 5 min. This suspension was filtered again and washed 3 times in phosphate buffer and one final wash of cold distilled water. Then, solvent extraction were made with one volume of chloroform:methanol (1:1) and three washes of 2 volumes of acetone until all colour was removed from the plant material. The resulting plant cell wall was then left overnight at room temperature and stored desiccated at 4 °C until required.

2. 2. 2. 2 Droplet-Inoculated and Brush-Inoculated Detached Leaves

The system of detached seedling leaves mounted on benzimidazole agar (Benedikz *et al.*, 1981) was used. A stock solution of benzimidazole (Sigma) in dimethylsulfoxide (DMSO, Sigma) was prepared. From this stock, an aliquot was added to molten 1% agar in distilled water, to give a final concentration of 150 p.p.m. benzimidazole. The agar-benzimidazole solution was dispensed into 10x10x1.5 cm non-sterile transparent square plastic boxes, then allowed to set. Seedling leaves were harvested by cutting the plants at soil level then washed in distilled water containing 0.02% Tween 20 (BDH). The distal 1 cm portions of each leaf were discarded and the next 5-cm segment cut and

mounted adaxial side up on the agar surface. Up to 12 leaf segments were mounted in each box.

Inoculum from sporulating cultures of *S. nodorum* was adjusted to $2x10^7$ spores /ml and brushed in one layer over the leaves. Alternatively, drops of 5 µl were inoculated ensuring that the pipette tip did not touch the surface of the leaf. Controls for these assays consisted of sterile distilled water containing 0.02% Tween 20, applied by brushing or as 5 µl drops, respectively.

The boxes were sealed with parafilm (to maintain high relative humidity), and incubated in a controlled environment cabinet. Conditions were adjusted to 16 hours photoperiod at 18-20 °C. Spore germination was assessed by placing the same spore suspension on clean glass slides in a humid chamber, created by placing moist tissue paper in Petri dishes. After 24 h in the same growth chamber as the leaves, germination was examined under light microscope.

2. 2. 3 Time Course Infection Experiments

Drop inoculum and controls were taken at 12, 24, 48 and 72 hours, pooled in 1.5 ml plastic tubes and flash frozen in liquid nitrogen and stored at -70 °C until required. Brush-inoculated leaves and controls were harvested at 2, 4 and 6 days and extracted in two different ways either for total protein extraction or for intercellular washing fluid.

2. 2. 3. 1 Total Protein Extraction

Harvested plant material was flash frozen in liquid nitrogen and stored at -70 °C until required. Frozen plant material was ground in liquid nitrogen, with an autoclaved, pre-cooled pestle and mortar. The powder obtained was transferred to a sterile centrifuge tube, weighed and mixed with cold extraction buffer. This buffer was 0.025 M sodium

phosphate, 0.2 M sodium chloride, 5% (w/v) polyvinyl pyrrolidone (PVP, Sigma) and 5 mM 2-mercaptoethanol (appendix 2). The proportion was 1g of plant material per 5 ml of buffer. The mixture was shaken on ice for 15 min and then spun at 4 °C at 8000xg for 10 min. The supernatants were transferred to autoclaved dialysis tubing and dialysed over night at 4°C, against buffer 10 mM citrate pH 6. The samples were concentrated by placing the dialysis tubing in polyethyleneglycol (PEG 35,000, Fluka) beds until reaching a final approximate concentration of 1g of plant material per 1ml of buffer. These extracts were stored at 4 °C for assays.

2. 2. 3. 2 Extracellular proteins in the IWF

The procedure described by Aked and Hall (1993) was followed. Wheat leaves were vacuum-infiltrated immediately after harvest, with cold extraction buffer (appendix 2) without PVP. Infiltrated leaves were cut in the middle and the intercellular washing fluid (IWF) was extracted by centrifugation at 2000xg for 5 min inside a 10-ml syringe. The supernatants were filtered through a 0.22μ membrane, dialysed, concentrated as above and aliquots were frozen at -70 °C before analysis.

2. 2. 4 Fungal Liquid Cultures with Wheat Cell Walls as a Single Carbon Source for Studying Secreted CWDE

For liquid cultures, an established biomass (see 2.2.1.2) was inoculated into 50 ml conical flasks containing 20 ml of basal salts liquid medium plus trace elements solution (appendix 2) and 1 % (w/v) wheat cell walls.

Three flasks of each treatment were inoculated and placed in an orbital incubator at 25°C at 125 rpm for 12, 24, 48, 72 and 120 hours. Culture fluids were first filtered through muslin and centrifuged at 10,000xg for 10 min to remove any fungus and then filtered

again through membranes following the sequence 5 μm, 1 μm and finally 0.44 μm to remove particulate material. The clear supernatants were frozen and stored at -70 °C.

2. 2. 5 Determination of Protein Concentration and Enzyme Assays

The protein content of each sample was determined using a modified Bradford protocol (Stoscheck, 1990.). A series of standard protein reactions was set up by pipetting 20 μl of protein solution (concentration from 0 to 30 μg), 50 μl of NaOH (1M) and 1ml of dye reagent in 1.5 ml microfuge tube. Finally, 200 μl was removed to a microtitre plate and the absorbance read at 595 nm on a Dynatech MR 5000 plate reader against the appropriate blanks A calibration curve was constructed using bovine serum albumin (BSA, Sigma), *Trichoderma* cellulase (TC, Fluka) and lysozyme (LY, Sigma) as standards (appendix 3).

2. 2. 5. 1 Reducing Sugar Assay

Some polysaccharide-degrading enzymes activities i.e. polygalacturonase (PG), xylanase (XY) and laminarinase (LA), were assayed from liquid cultures and plant extracts by measuring the release of reducing groups from corresponding polymer substrates, according to Somogyi's procedure (Somogyi, 1952, appendix 4).

Reaction mixtures contained 50 μl enzyme test solution and 250 μl of 0.1% substrate dissolved in 50 mM sodium acetate pH 5.5. Substrates (all from Sigma) were polygalacturonic acid, birch wood xylan and laminarin respectively. Three replicates and controls (to measure background sugar) were made for each test sample. The reactions were incubated at 42 °C for 15 min. The reactions were stopped by transferring reaction tubes into a boiling bath and then 250 μl of Somogyi's reagents 1 and 2 were added. The tubes were mixed, capped and transferred to a 95 °C water bath for 30 min. Later they were cooled before the addition of 250 μl of Nelson reagent. The tubes were then

vortexed and left overnight. Finally, 200 µl was removed to a microtitre plate and the absorbance read at 596 nm on a Dynatech MR 5000 plate reader against acetate buffer blanks. Standard curves were produced using known concentrations of the sugar monomers xylose, galacturonic acid and glucose respectively, in the range 0-100 µg. Enzyme activities were converted to nano katals (nKats; appendix 5).

2. 2. 5. 2 Viscometry Assay for Endo-PG and Endo-Xylanase Activities

Endo-polygalacturonase activity (endo-PG) was determined by viscometric assay, measuring the decrease in viscosity over time of 2 ml 5% (w/v) polygalacturonic acid (Sigma) in 50 mM citrate-phosphate buffer pH 5 and 400 µl of dialysed enzyme solution or boiled control, contained in a 3 ml viscometer at 25 ±1°C.

Endo-xylanase activity was also determined in the same 3 ml capillary viscometer, containing 2 ml of 5% (w/v) birch wood xylan (Sigma) in 50 mM citrate-phosphate buffer pH 5 and 400 µl of enzyme test sample or boiled control at 25 ± 1 °C.

Viscosity was measured at convenient intervals. Ten viscosity readings were obtained in each case and fitted in a designed computer programme that gave the equation curve and point for 20% reduction in relative viscosity. Activities were expressed as relative viscometric units (RVU), defined as 1000 / t_{20} time (min) for 20% decrease in relative viscosity.

2. 2. 5. 3 Xylanase assay vs Remazol Brilliant Blue Xylan

Xylanase activity was assayed spectrophotometrically by measuring the release of dye from the chromogenic substrate Remazol Brilliant Blue dye xylan (Sigma). The reaction mixture consisted of 5 to 20 µl enzyme test solution and 180 µl of 0.1% Remazol Brilliant Blue xylan dissolved in 50 mM sodium acetate pH 5.0.

Three replicates and three-boiled enzyme controls were made for each test sample. Reactions were incubated at 37°C for 30 min. and stopped by the addition of 500 µl of 96% ethanol. Tubes were then mixed and left to stand for 30 min before centrifugation at 11600 x g for 2 min, then 200 µl of the supernatant was removed to a microtitre plate and the absorbance read at 595 nm on a Dynatech MR 5000 plate reader against acetate buffer blanks.

2. 2. 5. 4 Glycosidases and Acetyl Esterase Assays

Glycosidases and acetyl esterase activities were assayed from liquid cultures and plant extracts by measuring the increase in absorbance resulting from the release of *p*-nitrophenol from their respective *p*-nitrophenyl derivative substrate (all from Sigma): β-D-glucopyranoside, β-D-xylopyranoside, β-D-galactopyranoside, β-D-glucuronide, α-L-arabinofuranoside for glycosidases and p-nitrophenol acetate for acetyl esterase. All *p*-nitrophenol derivates were dissolved in 50 mM sodium acetate pH 5.0 at 5 mM.

Each reaction mixture consisted of 100 µl of buffered glycoside solution and 10 µl of enzyme test solution or boiled control. Reactions were incubated at 42°C for 30 min and then stopped by the addition of 200 µl of 6% (w/v) $NaHC0_3$. Absorbance was read on a Dynatech MR 5000-plate reader at 405 nm against sodium acetate blanks. A calibration curve of *p*-nitrophenol was made in the range 0-5 µmoles, which was used to transform absorbances to enzyme activities in nano katals (appendix 5).

2. 2. 6 Electrophoretic Techniques

For the analysis of xylanase and arabinosidase isoforms, preparative and analytical isolectric focusing of liquid cultures, plant extracts, IWF and droplets were carried out.

2. 2. 6. 1 Preparative Isoelectricfocusing

Crude enzyme solutions obtained from culture filtrates and from tissue extracts from healthy and diseased plants were subjected to preparative isoelectric focusing using the Rotofor® Cell (BioRad). The electrodes were prepared by the addition of 25 ml of 0.1M NaOH and 25 ml of 0.1M H_3PO_4 respectively to the cathode and anode. The unit was pre-run with sterile distilled water prior to loading the sample, at low power conditions (<5W) for 5 min. The unit was drained using the harvesting apparatus and the procedure repeated until the current was 1-2 mA, this way insuring that excess ions were removed from both the cell and the surface of the ion exchange membranes; this procedure enabled increased starting voltage and better resolution during the focusing.

Loading of the sample was accomplished by injecting the mixture of 500 µl ampholytes (2% Bio-Lyte, pH 3-10, BioRad), the protein samples (400 µg) and a volume of sterile distilled water up to 55 ml, into the focusing chamber. The Rotofor was run at 12 Watts fixed power at 4°C. Voltage was recorded every 30 min until it stabilised (typically 3 hours), at which point 20 fractions (2.5 ml) were collected. pH of each fraction was recorded and protein content and enzyme activities were determined. Refractionation was accomplished after analysing the fractions from the initial separation. The relevant fractions were diluted with distilled water to 55 ml and reloaded in the Rotofor cell, run and collected as above. Since additional ampholytes are not added to the refractionation, the pH range of the refractionation corresponded to the pH range of the ampholytes contained in the pooled samples, creating a narrower range pH gradient and permitting better separation.

2. 2. 6. 2 Analytical IEF in Polyacrylamide Gels

Aliquots of culture filtrates, total leaf proteins, intercellular wash fluids (IWF) and droplets, containing 5 to 50 µg of protein were lyophilised, resuspended in 5 to 10µl of MilliQ water and subjected to analytical IEF and isoenzyme determination.

Precast polyacrylamide gels (Pharmacia) were used, with a pH gradient from 3.5 to 9.5 and IEF calibration kit (Pharmacia) on a LKB Bromma 2117 Multiphor horizontal electrophoresis apparatus, following manufacturer's instructions.

The preparatory process before running was as follows: the thermostatic circulator was set to 10°C for 20 min. The wells either side of the electrophoresis tank were filled with freshly prepared 1M NaOH in order to reduce the CO_2 in contact with the gel which may cause gradient drift in the basic region. A small volume (1-2ml) of liquid paraffin was applied to the electrophoresis plate and the screen print guide was smoothed onto the surface avoiding formation of air bubbles. An additional 1-2ml of liquid paraffin was placed on the guide print and the PAG plate carefully positioned above the guide, again ensuring no bubbles were present. The electrode strips were soaked evenly; cathode solution was 1M NaOH and anode solution was 1M H_3PO_4.The cathode and anode strips were cut to the exact size of the gel and laid in position. Application pieces were placed on the gel at position 3 to which samples, resuspended in 5 to 10 µl MilliQ water, were added.

The gel was run at 1500V, 50mA and 30 W for 45 min. The sample application papers were then removed and the gel run for a further 45 min. Care was taken to check the gel every 10-20 min and remove any condensation on the cover in order to avoid short circuits. Following separation, zymograms of the gel were obtained by substrate overlay gels of xylan and arabinose derivative, while marker proteins bands were stained.

Polyacrylamide gels (PAG) were also self-prepared, using the pre-dissolved acrylamide-bisacrylamide solution PlusOne ReadySol IEF T40 C3 (Pharmacia-Biotech) and ampholytes in the ranges from pH 2 to 7 and pH 3 to 8 [Pharmalyte, (Pharmacia-Biotech), 2.5/5.0 and Bio-Lyte, (Bio-Rad) 5.0/7.0 (40%); Bio-Lyte 3/5(20%) and 5/8(40%)]. Typically, to cast one 125x125x0.5 mm polyacrylamide gel (to run 12 samples), 10 ml solution was enough. The gel solution was prepared by mixing the components in the

proportion and order shown in appendix 6A. Preparatory process and running were as described above

Vertical IEF was accomplished by preparing gels using the Mini Protean electrophoresis casting plates and system of BioRad, in the pH gradient from 2.5 to 7 [Pharmalyte, (Pharmacia-Biotech), 2.5/5.0 and Bio-Lyte, (Bio-Rad) 5.0/7.]. Concentration of acrylamide (PlusOne ReadySol IEF T40 C3) and other reagents were adjusted as before (appendix 6A). For the running, the upper reservoir of the electrophoresis apparatus was filled with cathode solution (20 mM lysine, 20mM arginine, 2M ethylenediamine; all from Sigma), and the lower reservoir was filled with the anode solution (20 mM aspartate, 20 mM glutamate; both from Sigma). Samples containing 50 μg of protein were mixed with glycerol (to a final concentration of 10% v/v) and traces of bromophenol blue (BDH), before loading and running at a constant voltage (110 V), until the dye was at the bottom edge of the gel.

2. 2. 6. 2. 1 Improved IEF Gels

Improvement of the technique was made by incorporating high quality Urea (Ultra-Pure, Sigma) at 6M in the gel. Appropriate amount of urea was first dissolved in MilliQ water and later ampholytes and acrylamide polymer were incorporated in the order and proportions shown in appendix 6B. After deaeration under vacuum for about 5 to 10 min, TEMED was added and the solution was cast between thin glass plates to give a gel T5 C3 of 125x125x0.4 mm dimension. After 3 h, the gel was removed from the casting mould and kept in a humid chamber (a lunch box with moist tissue) for another 2 h, time given to complete polymerisation.

Preparatory process and running were as described above for the LKB Bromma 2117 Multiphor horizontal electrophoresis apparatus, except for sample preparation and electrolytes. In this case, lyophilized samples were dissolved in 20 μl loading buffer composed of 6 M urea in MilliQ water, 2% Ampholytes (according to the pH range) and

2%(v/v) 2-mercaptoethanol, added at the last minute from stock. The freshly prepared solubilization solution was incubated for 2 h at room temperature, before loading and running as described. Electrolytes were 0.02M NaOH as the cathode solution and 0.05M sulphuric acid as the anode solution. Once the running finished, the gel was washed for 15 min with three changes of buffer 50 mM Sodium-acetate pH 5.5 and the enzymes were detected.

Composite agarose-acrylamide gels IEF were prepared following the method of Johansson and Hjerten (1974) on 125x125x0.5 mm glass plates, using IEF grade agarose (Pharmacia). The stock of linear polyacrylamide polymer was prepared by mixing equal volumes of 20% (w/v) acrylamide monomer solution (electrophoresis grade acrylamide, BioRad) containing 0.2% (v/v) *N,N,N',N'*-tetraethylmethylendiamine (TEMED), with 0.14% freshly prepared ammonium persulphate solution (both prepared in MilliQ water). The mixture was immediately transferred to dialysis tubing and left for 2 h at room temperature. The highly viscous solution obtained was then dialysed for 4 days against MilliQ water, which was changed twice daily. This dialysed viscous polyacrylamide monomer (DVPM) stock solution was concentrated on PEG to give final concentration of 10% acrylamide (w/v).

Gels were made by mixing 0.1 g agarose (agarose-IEF, Pharmacia), 1.2 g stock of DVPM 10% (w/v) and milliQ water to 10 ml, to give a gel solution 1% (w/v) agarose and 12% (w/v) DVPM (see appendix 6B). The solution was heated until a clear solution was obtained and then let to cool to 70 °C, when ampholytes in the range 2.5-7.0 were added to give a final concentration of 2% (w/v). The solution was immediately poured between prewarmed glass plates and allowed to set. The gel was removed and kept in a humid chamber at 4 °C overnight.

Preparatory process and running were as described previously for the LKB Bromma 2117 Multiphor horizontal electrophoresis apparatus, using 0.1M NaOH as the cathode solution and 0.04M aspartic acid as the anode solution.

2. 2. 6. 2. 2 Determination of Protein Isoelectric Point.

Sections of the gels containing 10µl of protein markers (Broad range pI markers, Pharmacia) were cut and pieces fixed for 60 min in 10%(w/v), trichloroacetic acid (Sigma), 4% (w/v) 5-sulphosalicylic acid (Sigma) and 40% (v/v) methanol. After washing for 20 min (with two changes) in destaining solution [25% (v/v)] ethanol, 8% (v/v) acetic acid], markers were stained for 15 min in 0.5% (w/v) Coomassie Blue R-250 (Sigma) dissolved in destaining solution. Finally, pieces were added to several changes of destaining solution until background became clear. For estimation of pI, the distance from the cathode of each pI marker protein was measured and plotted against their correspondent known pI (appendix 7). The distance from the cathode of the proteins of interest was then measured and converted into pI with the equation of the curve.

2. 2. 6. 2. 3 Zymograms

Xylanase activity was detected following the method of Mackenzie & Williams (1984), by overlaying the protein gel with a substrate gel, composed of 0.1%(w/v) birchwood xylan (Sigma) thoroughly dissolved in buffer 100 mM sodium acetate pH 5.5 and 1.5%(w/v) agarose. The solution was boiled until agarose was dissolved and then cast into a preheated glass cassette with 0.75-mm spacers. The gel was let to set for at least 1 h at 4°C. The overlay was applied taking care to remove all air bubbles and ensuring close contact. Incubation was at 50°C for 20 min after which the substrate gel was removed and washed with 1M NaCl for 10 min and stained with 0.1% Congo Red (Sigma) for 20 min. Clear zones of hydrolysis were visualised by destaining with 1M NaCl for 1 h.

The method of Biely *et al.* (1985) was also used, overlay gels contained 0.5%(w/v) Remazol Brilliant Blue -Xylan (RBB) and 1.5% (w/v) agarose dissolved in buffer 100 mM sodium-acetate pH 5.5. To cast the substrate gel, the dye was dissolved in half

volume of buffer and hated on a water bath at 70°C. Agarose was dissolved in the other half volume and brought to boil until a clear solution was obtained.

The, two solutions were then mixed thoroughly and poured into a preheated glass cassette with 0.75 mm spacers. The gel was let to set for at least 1 h at 4 °C. The overlay was applied taking care to remove all air bubbles and ensuring close contact. Incubation was at 40°C for 1 hour after which the overlay gel was destained by extensive washing in 50% (v/v) ethanol in buffer 50 mM acetate pH 5.5 for 3 to 20 h.

Detection of arabinofuranosidase activity was performed following the method of Fernandez-Espinar *et al.* (1994), immersing the gel into buffer 20 mM succinate containing 1 mM 4-methylumbelliferyl α-L-arabinofuranoside and incubating at room temperature until bands appeared (5 to 10 min). The method of Morales *et al.* (1995) was also used, overlaying 1.5% agarose gels containing 3 mM p-nitrophenyl-α-L-arabinofuranoside in 50 mM acetate buffer pH 5.5. The sandwich was incubated for 15-20 min at room temperature and activity visualised as yellow bands in a colourless background after washing the gel with a solution of 6% (w/v) $NaHC0_3$.

2. 3 Results

Stagonospora nodorum culture supernatants and extracts of inoculated and control leaves, were assayed for different CWDE and the activities compared. This was with the aim of determining the potential enzymatic spectrum of *S. nodorum* and assigning one or some CWDE a possible key role in the pathogenicity of this fungus using the following criteria:

1. Their presence *in vitro*.
2. Their presence in infected tissues at higher levels than in controls.
3. Their presence at early stages of infection (before or coincident with symptoms) and correlation with increased expression during time course infection experiments and also *in vitro*.
4. Different levels of expression among isolates of different aggressivity.
5. Identification of fungal isoforms.

2. 3. 1 Expression of Polysaccharide-Degrading Enzymes *In Vivo* and *In Vitro*

Laminarinase (LM), polygalacturonase (PG) and xylanase (XY) activities, expressed as reducing sugars liberated from their respective substrates were determined.

The maximal activity for all depolymerases *in vitro* was observed after 72 hours of culture. Polygalacturonase showed the highest activity (10 nkats/ml) followed by xylanase (6 nkats/ml) and β-1,3-glucanase (3 nkats/ml). In extracts from inoculated leaves, β-1,3-glucanase was lower than controls (healthy leaves) by the end of the period tested, while polygalacturonase activity increased transiently, then returned to levels similar to controls. Xylanase levels remained low throughout in healthy leaves, but of all activities, xylanase was the only one that showed sustained significant high levels during

all the period tested, in culture filtrates as well as in extracts from inoculated leaves, as shown in Fig 8.

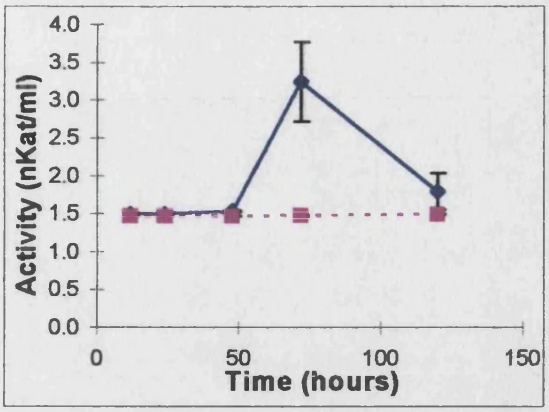
Since the substrates used for reducing groups are heteropolymers containing different appending linkages that could appear as *apparent* polysaccharidase activities and because of lack of specific endo-activity detection, a viscometric assay was done for xylanase and polygalacturonase activities. Endopolygalacturonase activity in *vitro* showed an early peak (24 h) but returned to levels of the control leaves at the end of the period tested (Fig 9a). *In vivo*, PG activities of treatments were very close to those of controls (Fig 9b). Endoxylanase activity *in vitro* and *in planta* was detected at higher levels in treatments than in controls, reflecting the pattern shown by the previous assay. In inoculated leaf extracts, xylanase increased steadily, until the end of the test period at 120 h (Figures 9c and 9d).

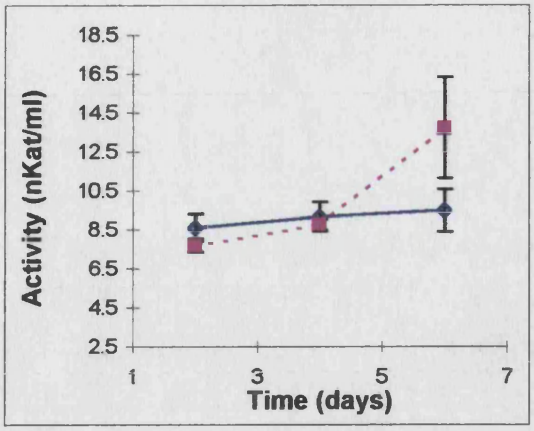
Endo-xylanase activity was also tested using the Remazol Brilliant Blue xylan assay. This was done because of the specificity of this method in detecting endoxylanase activity against a dye substrate-complex. It confirmed endo-xylanase activity *in vitro* (maximal at 72 h) and a similar pattern of increase *in planta* (Fig 10), as was revealed by reducing group assay and viscometry.

2. 3. 2 Expression of Glucosidases and Acetyl Esterases *In Vivo* and *In Vitro*

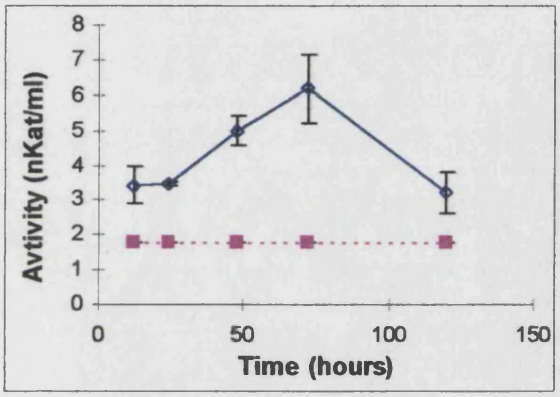
Enzymatic activities cleaving the side chains of arabinoxylans and pectin, arabinosidase, acetylesterase, glucosidase, glucuronidase, galactosidase and xylosidase, were detected in liquid cultures as well as in inoculated and healthy leaves (Figures 11 and 12).

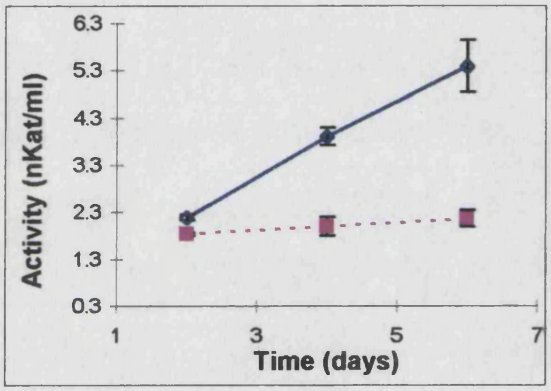
In culture filtrates, arabinosidase was the highest activity (15 nkats / ml, Fig 12e), followed by glucosidase (5 nkats/ml, Fig.11a), galactosidase (Fig. 11c) and xylosidase (Fig. 12c), with 1.5 nkats/ml. In contrast, glucuronidase (0.8 nkats/ml, Fig 11e) and acetylesterase (0.4 nkats/ml, Fig. 12a) were the lowest. Only arabinosidase (2 nkats/ml)



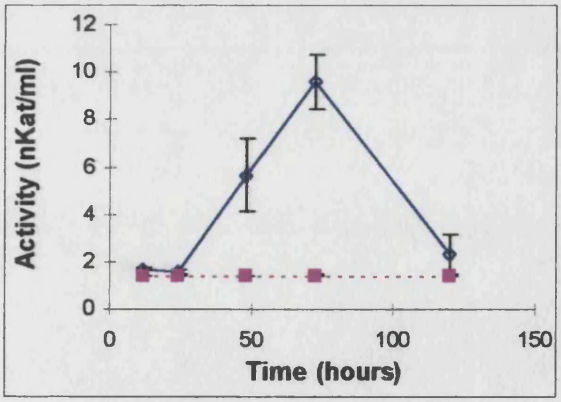


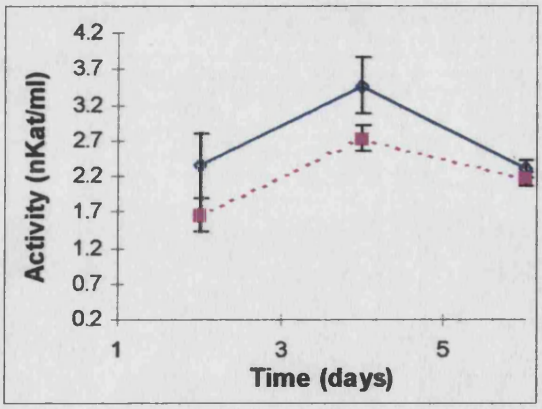
(a) Laminarinase in culture filtrates **(b)** Laminarinase in leaf extracts





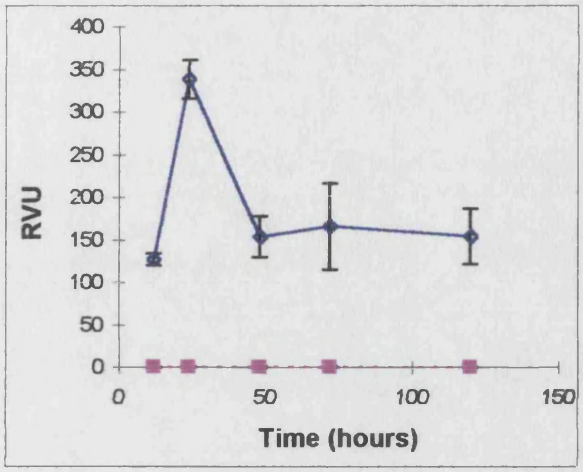
(c) Xylanase in culture filtrates **(d)** Xylanase in leaf extracts



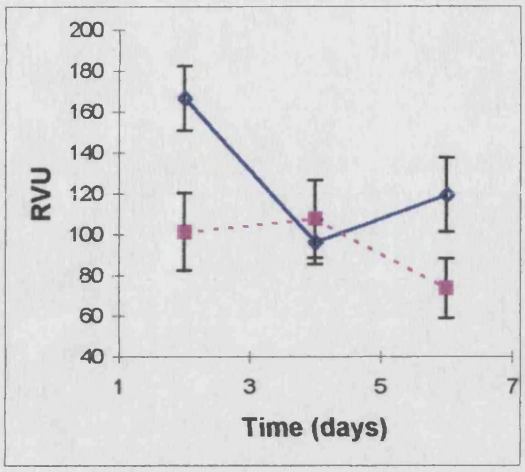


(e) Polygalacturonase in culture filtrates **(f)** Polygalacturonase in leaf extracts

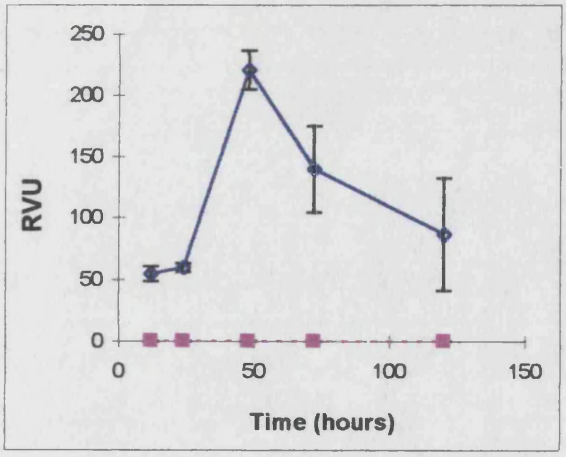
Figure 8: Polysaccharidase–degrading enzymes detected in culture filtrates (a, c and e) and inoculated leaves extracts (b, d and f). Controls were fungus growing in basal salts medium and healthy leaves respectively. Activity is expressed as nanomole of reducing sugars equivalents per second per ml (nKat/ml). Bars indicate standard deviation of three replicates. One representative experiment from three is shown.
(◇) is treatment, and (■ .) is control.



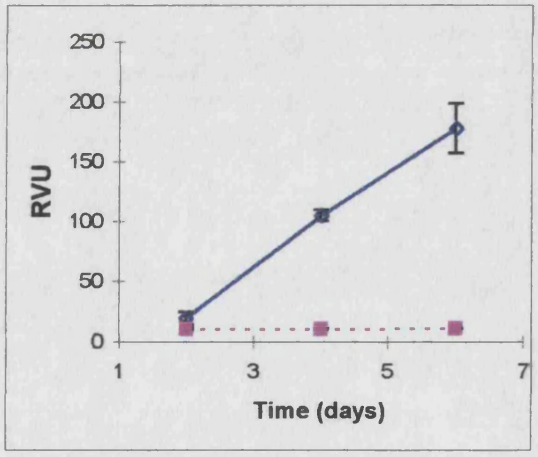
(a) Endopolygalacturonase in culture filtrates



(b) Endopolygalacturonase in inoculated leaf extracts

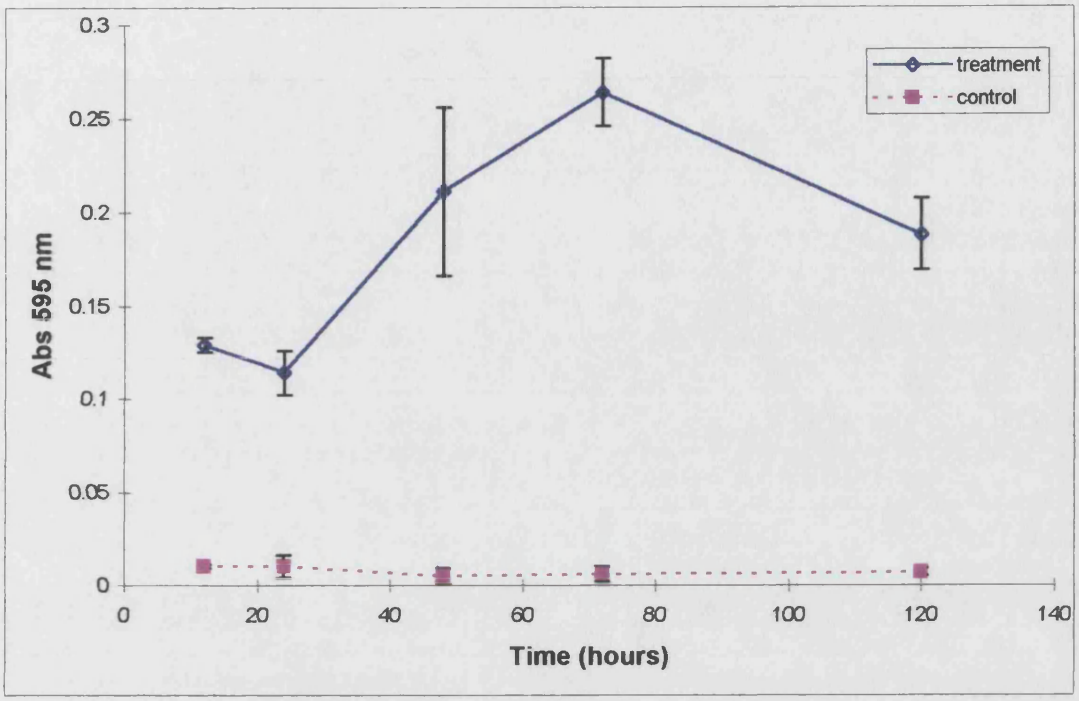


(c) Endoxylanase in culture filtrates

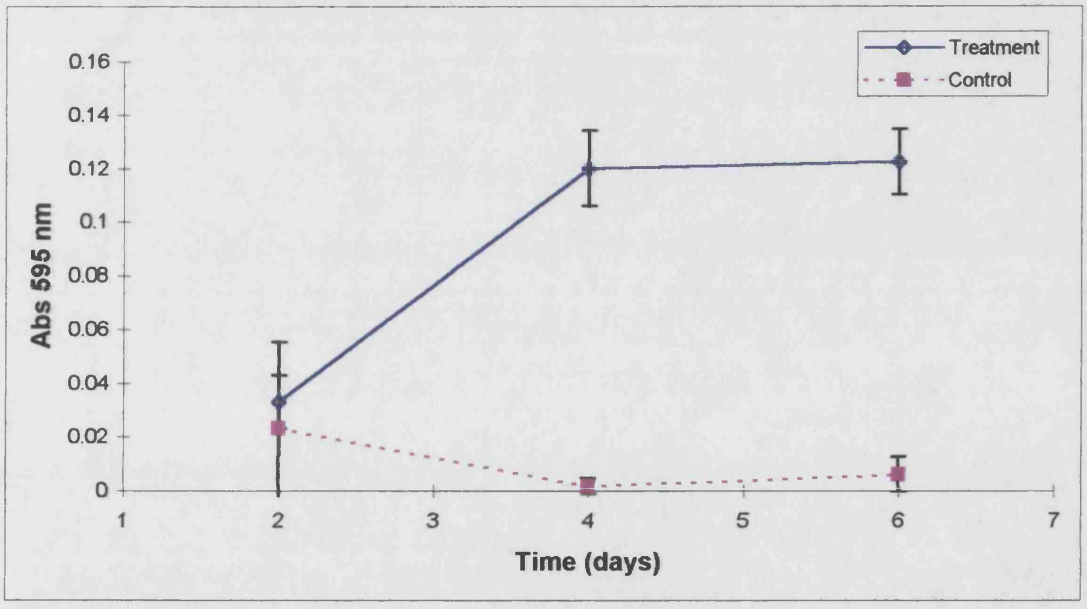


(d) Endoxylanase in inoculated leaf extracts

Figure 9 : Polysaccharidase–degrading enzymes detected in cultures filtrates (a and c) and inoculated leaves extracts (b and d). Controls were fungus growing in basal salts medium and healthy leaves. Activity is expressed as relative viscometric units (RVU). Bars indicate standard deviation of three replicates. One representative experiment of three is shown. (◇) is treatment, and (■) is control.

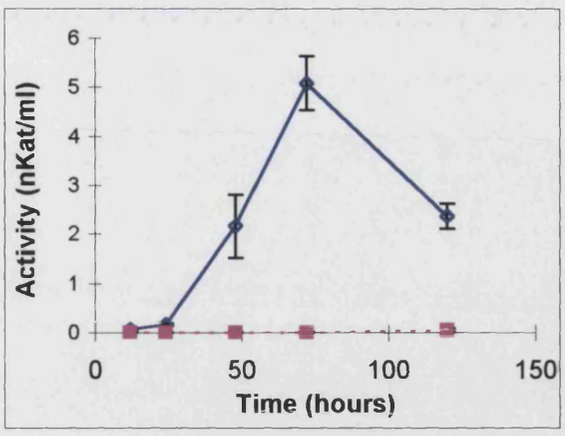


(a) Endoxylanase in culture filtrates

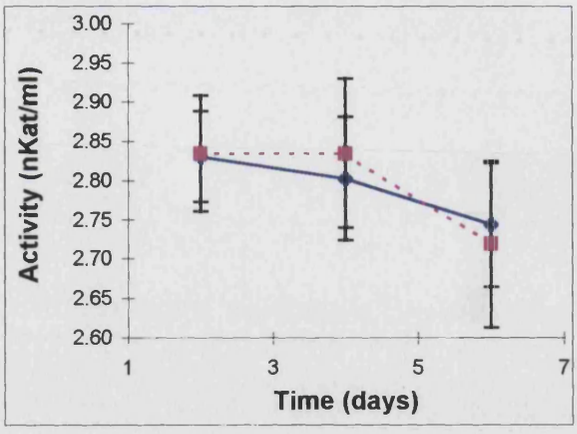


(b) Endoxylanase in inoculated leaf extracts

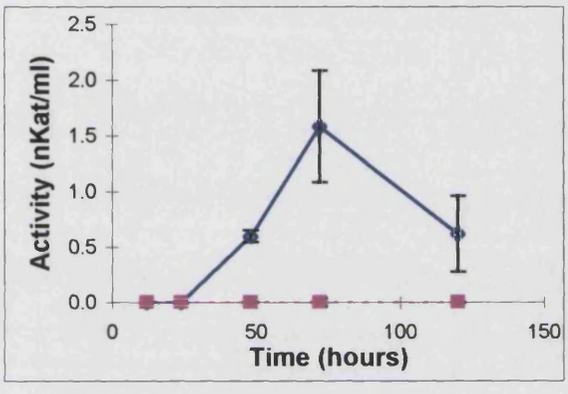
Figure 10 : Endoxylanase activity detected in (a) culture filtrates, and (b) extracts from inoculated leaves extracts. Controls were fungus growing in basal salts medium and healthy leaves respectively. Activity is expressed as absorbance at 595 nm. Bars indicate standard deviation of three replicates. One representative experiment from three is shown. (◇) is treatment, and (■) is control.



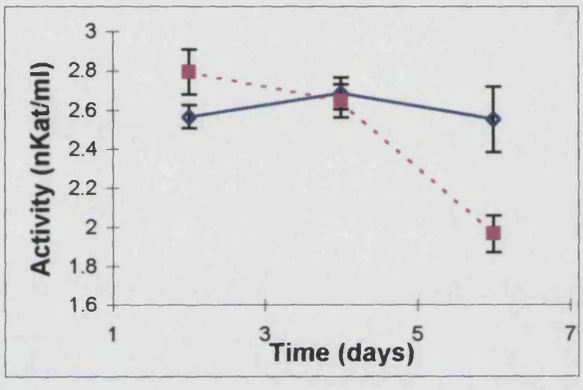
(a) β-D-glucosidase in culture filtrates



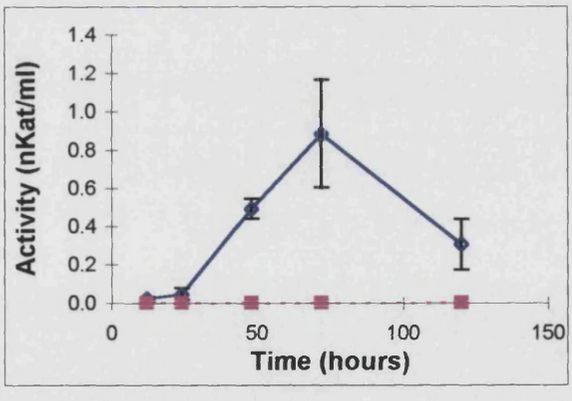
(b) β-D-glucosidase in inoculated leaf extracts



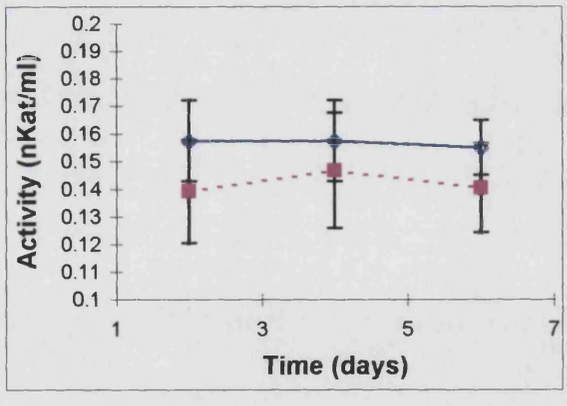
(c) β-D-galactosidase in culture filtrates



(d) β-D -galactosidase in inoculated leaf extracts

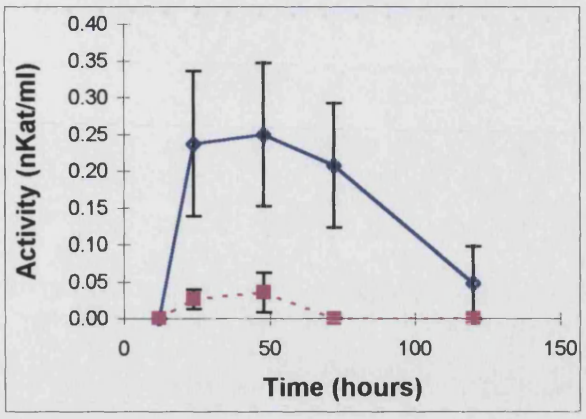


(e) β-D-glucuronidase in culture filtrates

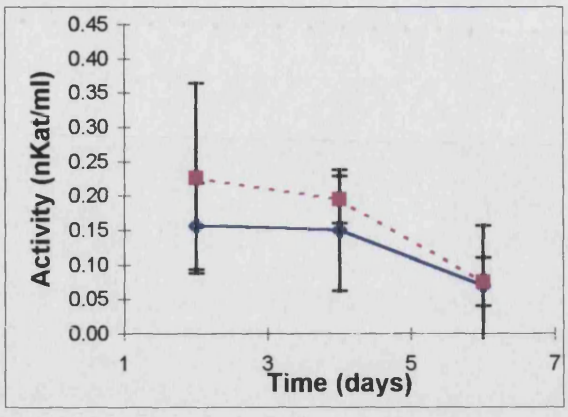


(f) β-D-glucuronidase in inoculated leaf extracts

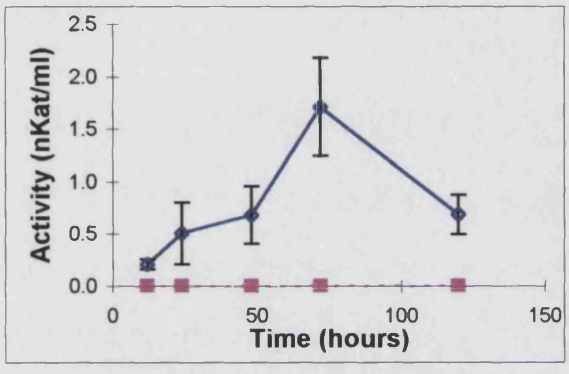
Figure 11 : Glycosidases detected in culture filtrate (a, c and e) and inoculated leaves extracts (b, d and f). Controls were fungus growing in basal salt medium and healthy leaves respectively. Activity is expressed as nanomole of reducing sugars equivalents per second per ml (nKat/ml). Bars indicate standard deviation of three replicates. One representative experiment from three is shown. (◇) is treatment, and (■) is control.



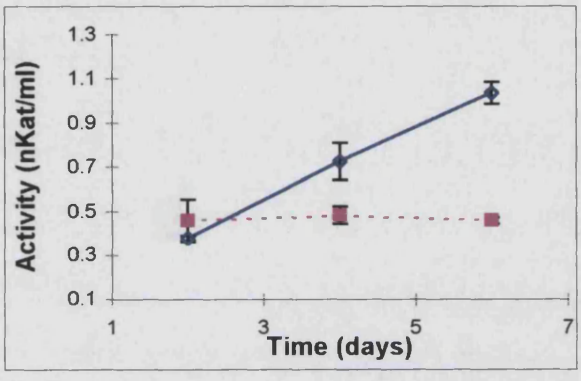
(a) Acetylesterase in culture filtrates



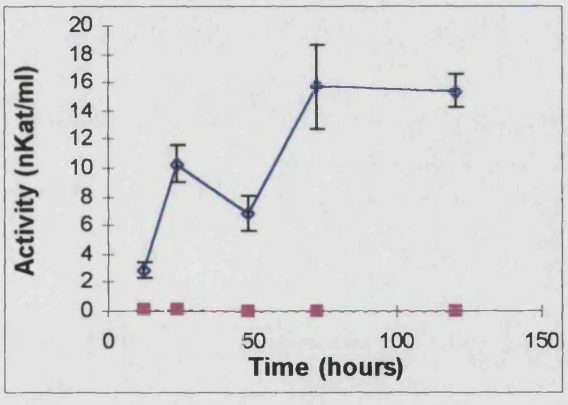
(b) Acetylesterase in inoculated leaf extracts



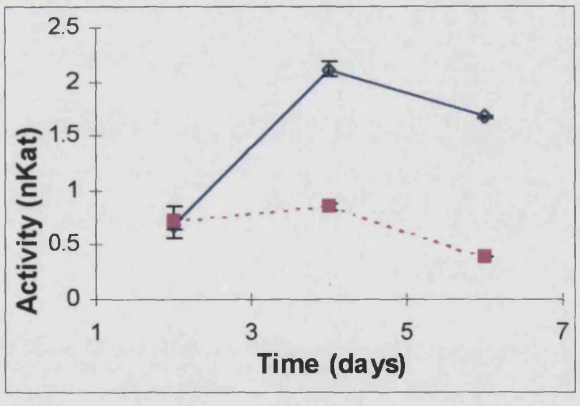
(c) β-D-xylosidase in culture filtrates



(d) β-D -xylosidase in inoculated leaf extracts



(e) α-L-arabinosidase in culture filtrates



(f) α-L-arabinosidase in inoculated leaf extracts

Figure 12 : Acetylesterase and other glycosidases detected in culture filtrates (a, c and e) and inoculated leaves extracts (b, d and f). Controls were fungus growing in basal salt medium and healthy leaves respectively. Activity is expressed as nano mole of reducing sugars equivalents per second per ml (nKat/ml). Bars indicate standard deviation of three replicates. One representative experiment from three is shown.
(◇) is treatment, and (■) is control.

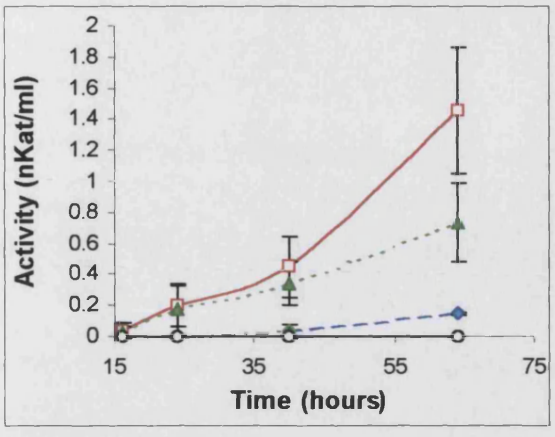
and xylosidase (1 nkats/ml) were consistently present in higher levels in inoculated leaves than in controls. Arabinosidase reached maximal level at 4 days post inoculation, whereas xylosidase was still increasing by 6 days.

2. 3. 3 Expression of Xylanase and Arabinosidase in the IWF and Droplets of Inoculated and Control Wheat Leaves

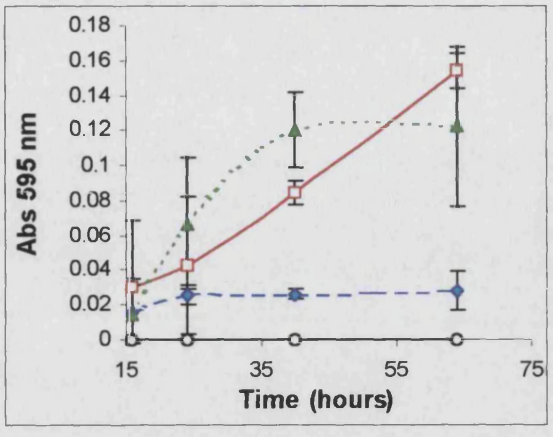
Among all the activities tested, xylanase and arabinosidase were chosen for further comparison between strains of different aggressiveness, based on the significance of arabinoxylans in cereal primary wall structure and on the predominant activities *in vitro* and in infected leaves. After inoculating leaves (see 2.2.2.2) with strains BS 471, BS 171 and LAW, a time course experiment was set up and droplets and leaves collected (see 2.2.3). Then, IWF was obtained (see 2.2.3.2) and xylanase and arabinosidase activities determined (see 2.2.5.3 and 2.2.5.4, respectively).

As Fig 13 shows, both arabinosidase and xylanase activities increased steadily during the time course experiments for the three strains. There was no detectable xylanase activity in any of the controls, and arabinosidase activity was absent from the control droplets. The IWF of control leaves did show arabinosidase activity but it was always lower, in the order of half that of inoculated leaves.

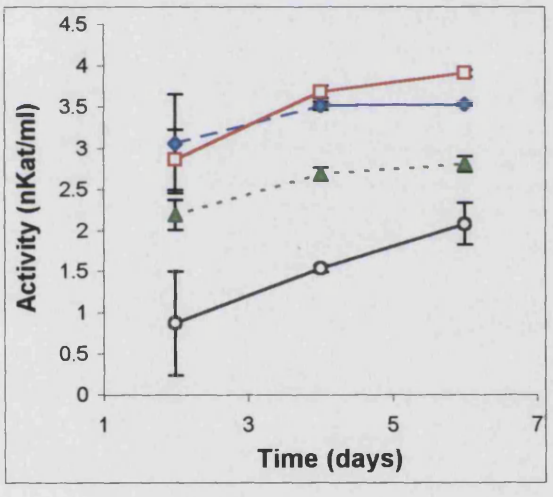
In both droplets and IWF, BS 471 showed the highest arabinosidase and xylanase activities. e.g. in the droplets: 1.4 nkats/ml and 0.15 units, respectively; and in the IWF 4 nkats/ml and 0.35 units, respectively. LAW showed the second higher arabinosidase and xylanase activities in the droplets, e.g., 0.8 nkats/ml and 0.12 units, respectively. However, BS 171 showed the second higher arabinosidase and xylanase activities in the IWF. e.g., nkats/ml and 0.2 units respectively.



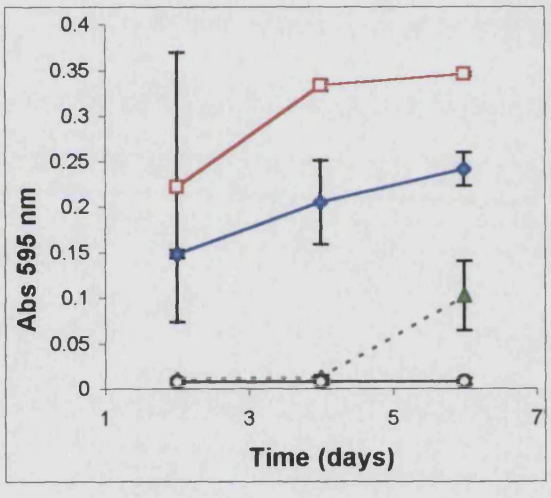
(a) α-L-arabinosidase activity in inoculation droplets



(b) β-xylanase activity in inoculation droplets



(c) α-L-arabinosidase activity in the IWF



(d) β-xylanase activity in the IWF

Figure 13 : Arabinosidase and xylanase activities detected in inoculation droplets (a and b) and IWF of inoculated leaves (c and d). Controls were solution of Tween 0.02% and healthy leaves, respectively. Activity is expressed as nanokatals / ml for arabinosidase and absorbance at 595 for xylanase activity over RBB-xylan. Bars indicate standard deviation of three replicates. One out of two experiments performed is shown. Strains used were : BS 471 (□); BS171 (◆); Law (▲); and (○)is control.

The fact that BS 471 showed the highest arabinosidase and xylanase activities was coincident with the aggressiveness of the strains; BS 471 was the most aggressive of the three as evident by production of symptoms earlier than BS 171 and LAW and the lesions spread more rapidly.

2. 3. 4 Influence of Carbon Source on Arabinosidase and Xylanase Production.

Strains BS 171 and BS 471 were cultured *in vitro* as described (see 2.2.4), using either cell walls, xylose or xylan as carbon sources. Filtered supernatants were taken and weighted and xylanase and arabinosidase activities determined as above. Comparison of the enzymatic activities secreted by these two isolates at 72 h, is shown in Table 1.

Table 1. Enzymatic activities per 100 mg fresh weight of hyphae, secreted by *S. nodorum* isolates BS 171 and BS 471 on wheat cell walls, xylose and xylan as carbon sources at 72 h of culture. Mean of three replicates ± standard deviation are shown. (1) = relative viscometric units (RVU); (2) = A 595 nm. Glycosidase activities are as nkats.

Enzymatic activity (n kat)	Cell Walls		Xylose		Xylan	
	BS171	BS471	BS171	BS471	BS171	BS471
arabinosidase	1.29±0.09	1.34±0.07	0.68±0.05	0.75±0.08	0.92±0.1	0.83±0.09
xylosidase	0.43±0.04	0.46±0.06	0.38±0.08	0.41±0.07	0.55±0.04	0.62±0.03
xylanase (1)	0.90±0.04	0.84±0.03	0.02±0.01	0.02±0.02	0.20±0.05	0.32±0.04
xylanase (2)	0.081±0.03	0.090±0.05	0.033±0.03	0.023±0.02	0.045±0.02	0.044±0.01

Wheat cell walls were the most effective inducer for xylanase and arabinosidase, whereas xylan was a more effective inducer of xylosidase. However, no clear indication of mode of regulation of these enzymes is apparent from these data. Non-substantial differences were

found when comparing levels of activities produced for each strain *in vitro*. Distinguishable characteristics among filtered supernatants were the viscosity and the slimy appearance, especially conspicuous for BS 471 cultures.

2. 3. 5 Arabinosidase and Xylanase Isoform Patterns

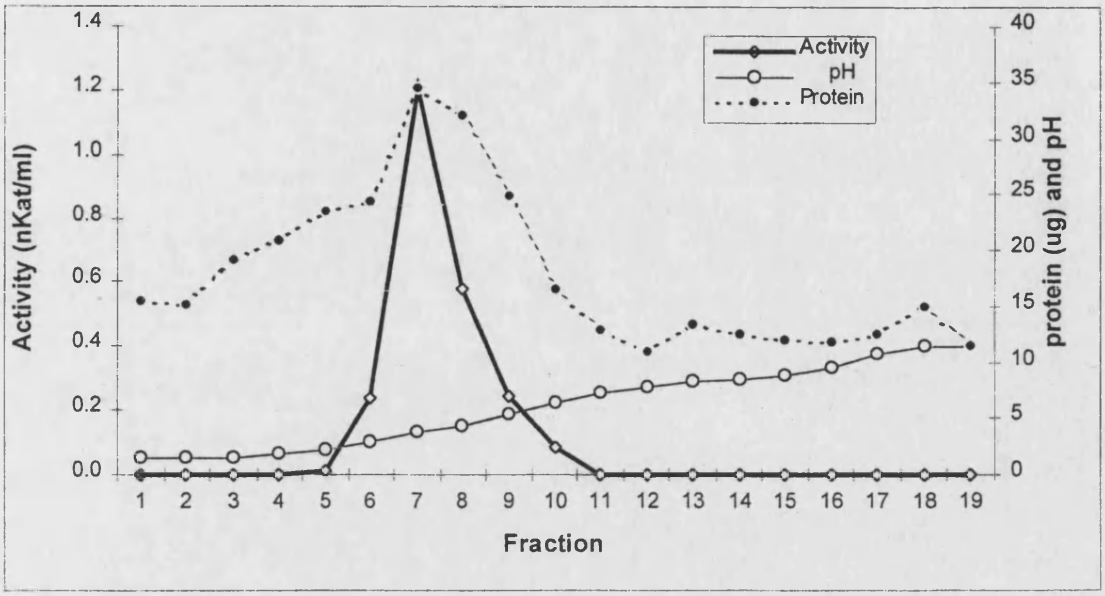
With the aim of correlating the occurrence of xylanase and arabinosidase with fungal growth within the host tissues and in liquid cultures, preparative and analytical isoelectricfocusing patterns of these proteins were obtained in both systems. Preparative electrophoresis was also used as a possible preliminary step in separation (purification) of these activities. Samples showing the maximal enzymatic activities were used, i.e. 72 h liquid cultures and total extracts of leaves 6 d after inoculation. Controls leaves were also included since it was attempted to reveal which isoforms were responsible for enhanced activities during infection.

IWF and droplets were used for analytical IEF for simplifying comparison, since it was expected that these samples contained less host protein than extracted whole leaves. Liquid cultures were also run together with IWF and droplets for comparison.

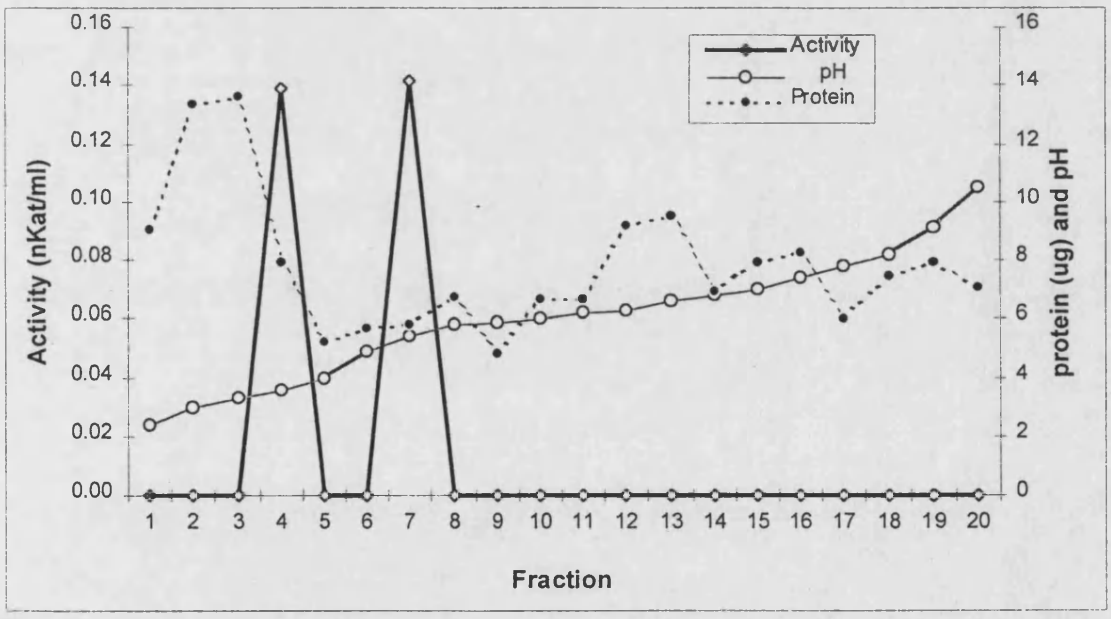
2. 3. 5. 1 *In Vivo* and *In Vitro* Isoform Patterns of Arabinosidase

Arabinosidase profiles in the Rotofor column from culture filtrates showed one wide peak (Fig 14), which was resolved into two different isoforms after refractionation. The pIs were 3.6 and 5.4 and activity of 0.14 nKats and 0.017 nKats respectively.

Patterns from extracts from infected and healthy leaves showed a wide peak of activities, contained in around eight fractions, in the pI range from 3.3 to 6.6 for infected leaves extracts, and in the range from 4.1 to 7.0 for control (Fig 15).

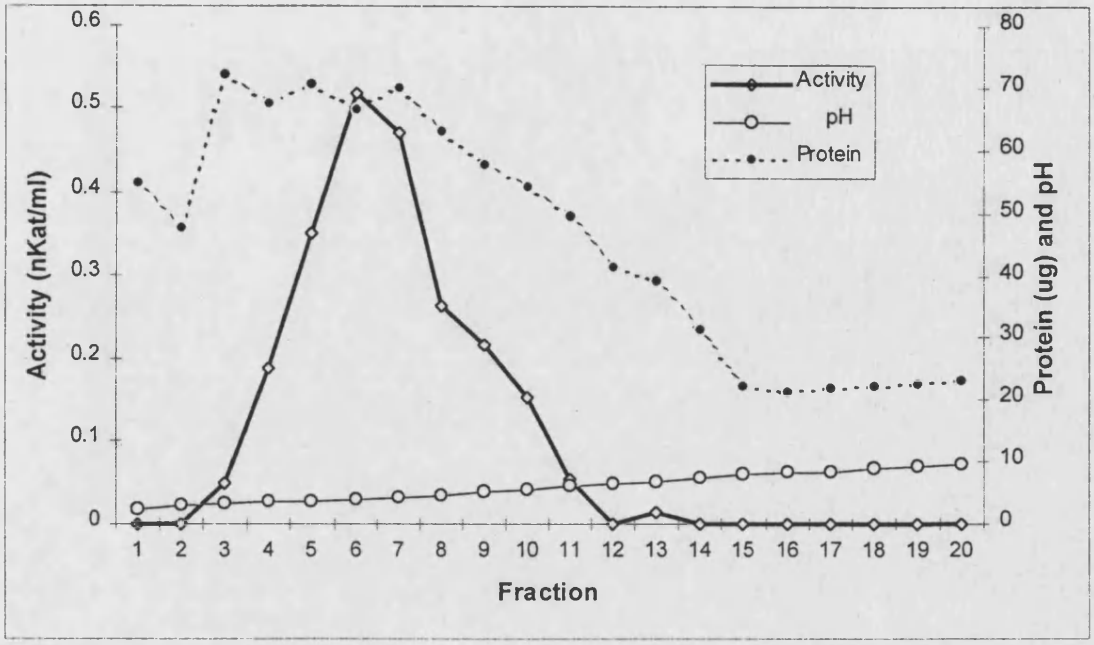


(a)

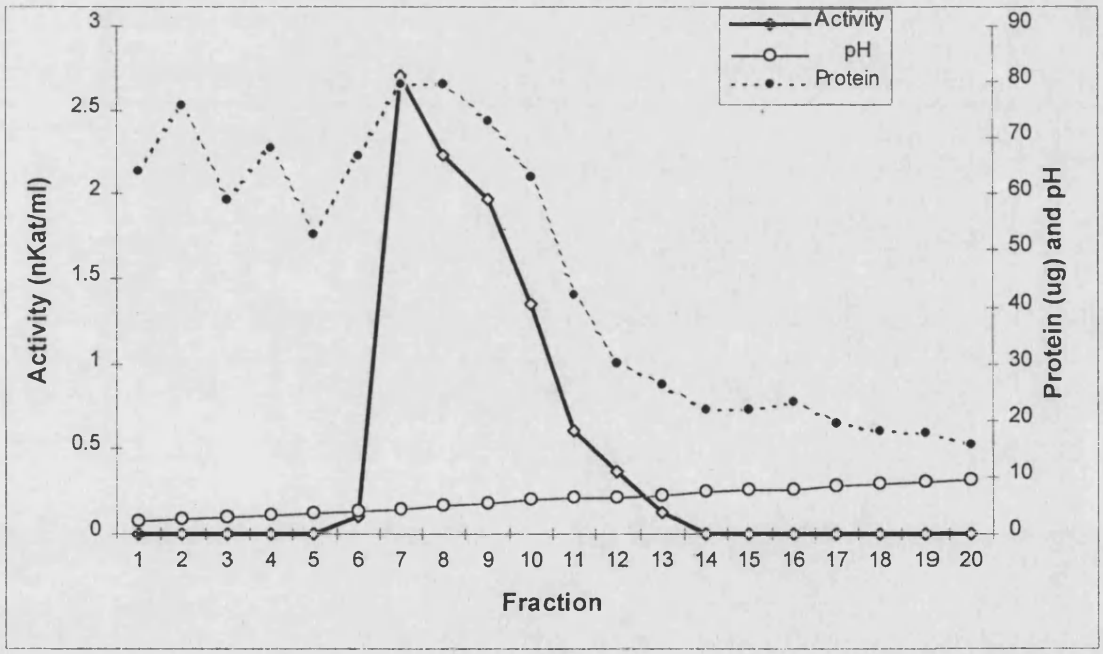


(b)

Figure 14 : Isoelectric focusing patterns of extracellular arabinosidase released by *S. nodorum* in culture filtrates, as detected by preparative IEF with the Rotofor system. (a) First running of liquid culture filtrate; (b) second running (refractionation) of liquid culture filtrate of fractions of the first run that showed arabinosidase activity (6 to 11). One out of two experiments performed is showed.



(a) Treated



(b) Control

Figure 15 : Rotofor isoelectric focusing patterns of arabinosidase in extracts from : (a) leaves infected with *S. nodorum*, and (b) control leaves.

Comparison of analytical IEF (pH 3 to 8) of culture filtrates and IWF in composite agarose gels using deglycosylated samples confirmed the presence of two arabinosidases, though smearing of the bands occurred (Fig 16a). The pI s of these isoforms were 5.0 and 4.0, which appeared in the culture filtrates and in the IWF and inoculating droplets of the strains BS 171 and BS 471 and Ln 97.

During the course of xylanase and arabinosidase purification, conditions for resolving better electrophoretograms were found (Chapter 3), including the use of 6M urea in the gels and the treatment of the samples with 6M urea and β-mercaptoethanol. Under these conditions, it was possible to resolve clearly two arabinosidase isoforms (pI's 4.5 and 3.9) in the IWF of inoculated plants, in inoculating droplets and the culture filtrate, as seen in Fig 17a. Three other isoforms were present in the IWF, which appear to be of host origin, since they were also present in the IWF of controls but not in inoculating droplets or in the liquid culture.

2. 3. 5. 2 *In Vivo* and *In Vitro* Isoform Patterns of Xylanase

Xylanase from culture fluids (Fig 18) showed a broad peak comprising fractions 4 to 12. Refractionation of these fractions (Fig 18b) did not achieve complete separation of peaks but a wide range of isoforms (fractions 5 to 19) were better visualised by the Remazol Brilliant Blue assay (Fig 18c), which revealed at least six isoforms with pIs of 5.0; 5.4; 6.0; 6.6; 7.4 and 9.0.

Xylanase patterns from extracts from infected and healthy leaves (Fig 19a and 19b) revealed one plant isoform (pI 2.4) and a group of at least four peaks present in the infected leaves extract.

Attempts to resolve single xylanase activity bands on the gels failed. Instead, smeared clearing zones were obtained. This was in spite of attempting several different treatments of the samples (e.g. ultrafiltration, molecular sieving, stabilisers), electrophoresis,

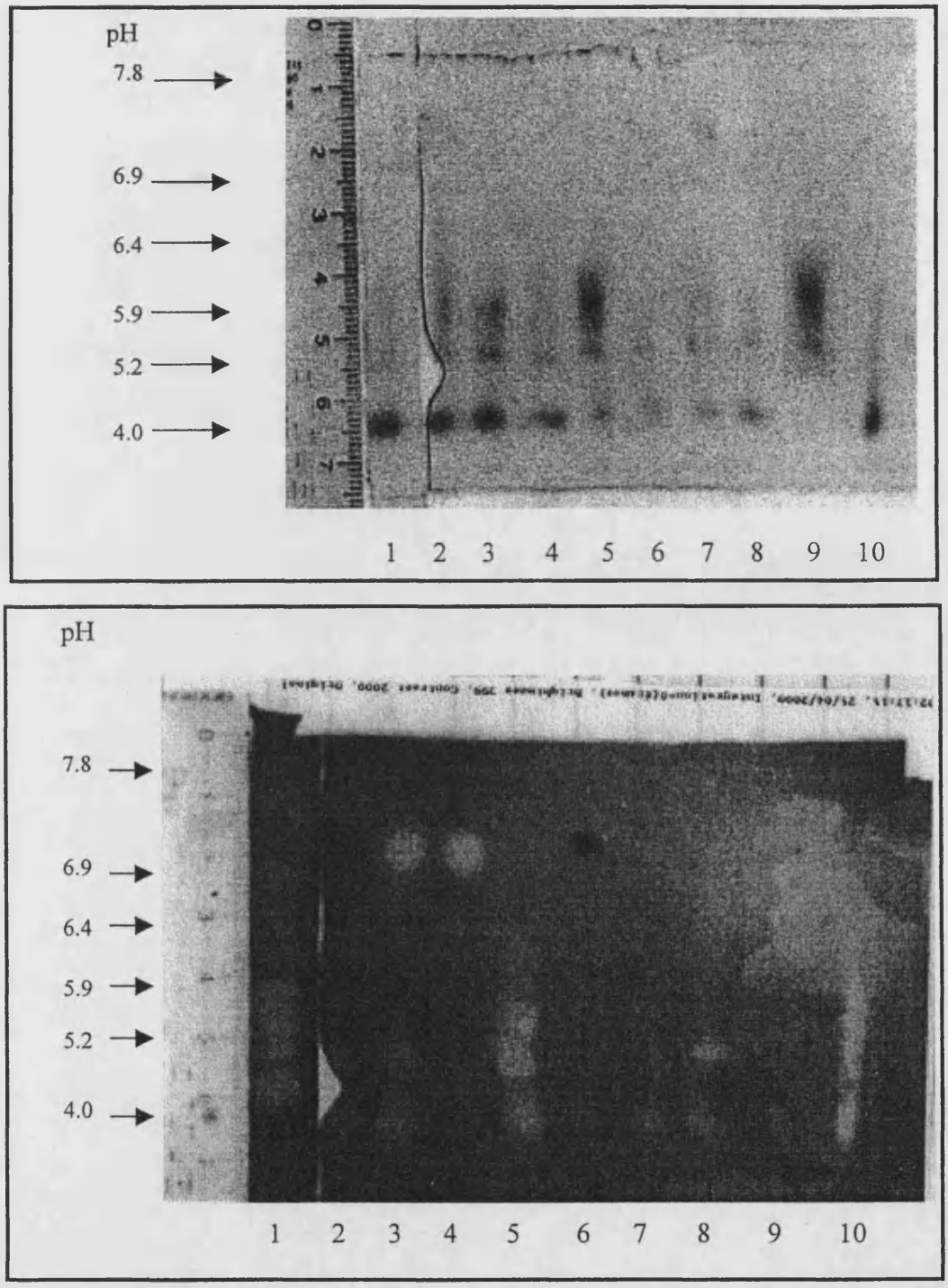


Figure 16 : Zymograms for (a) arabinosidase and (b) xylanase activities, using deglycosylated samples on composite agarose gels. **1:** culture filtrate; **2-5: IWF** at 4 and 6 days of 171 and 471 respectively; **6 - 8:** droplets at 64 hours of Ln97, 171 and 471 respectively; **9 :** control leaves and **10:** culture filtrate without deglycosylation treatment.

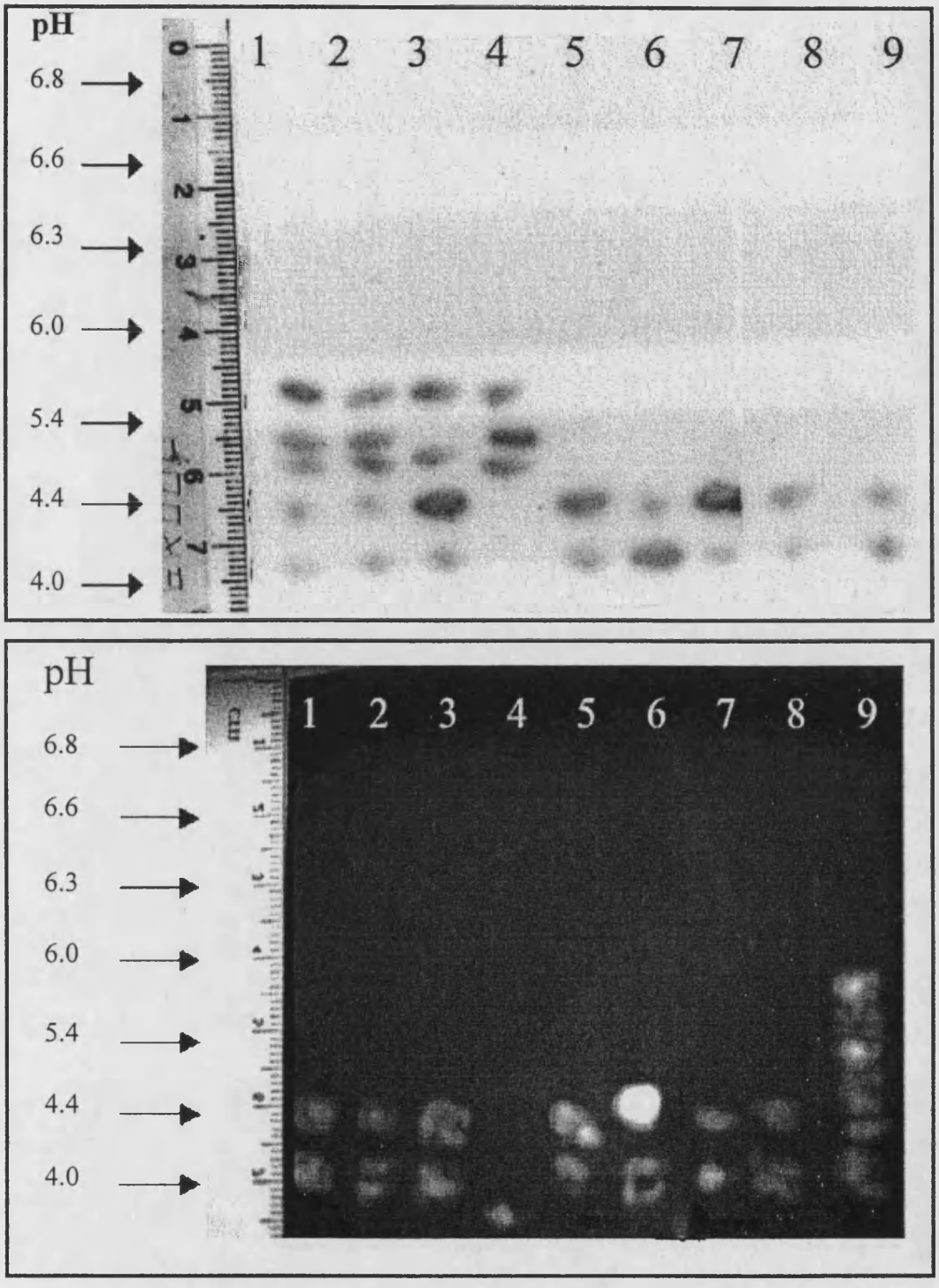
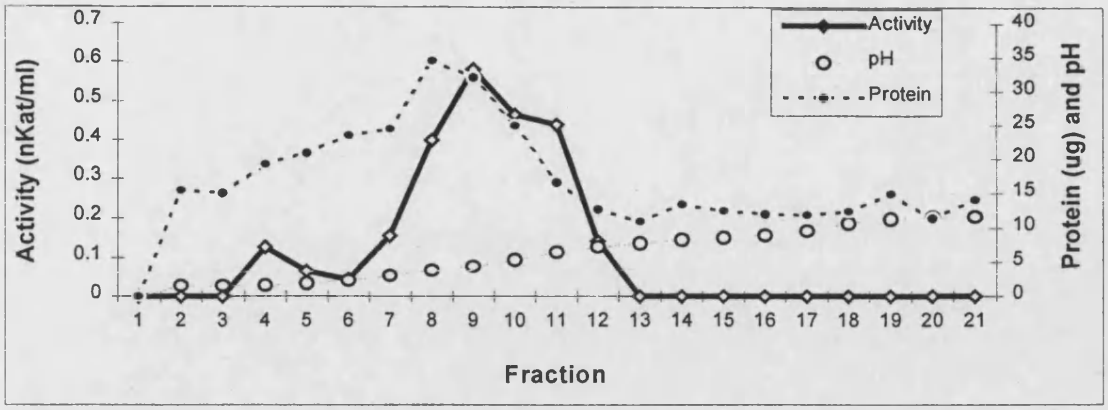
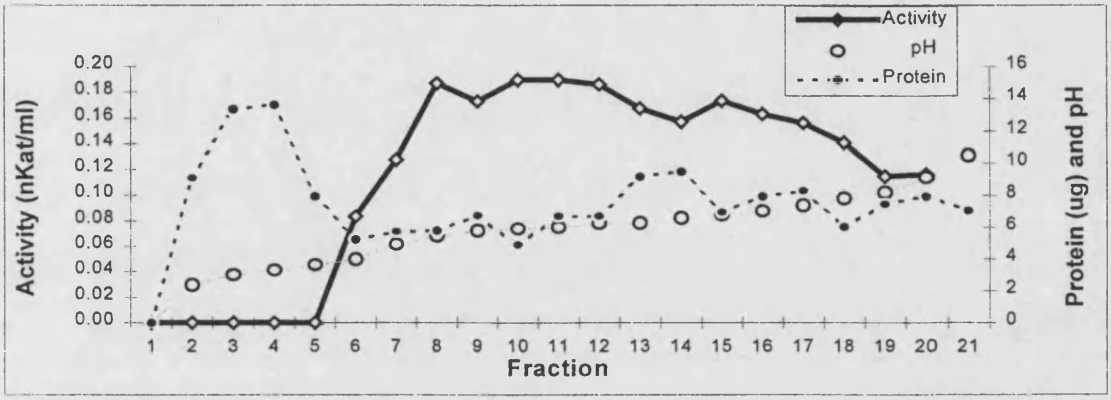


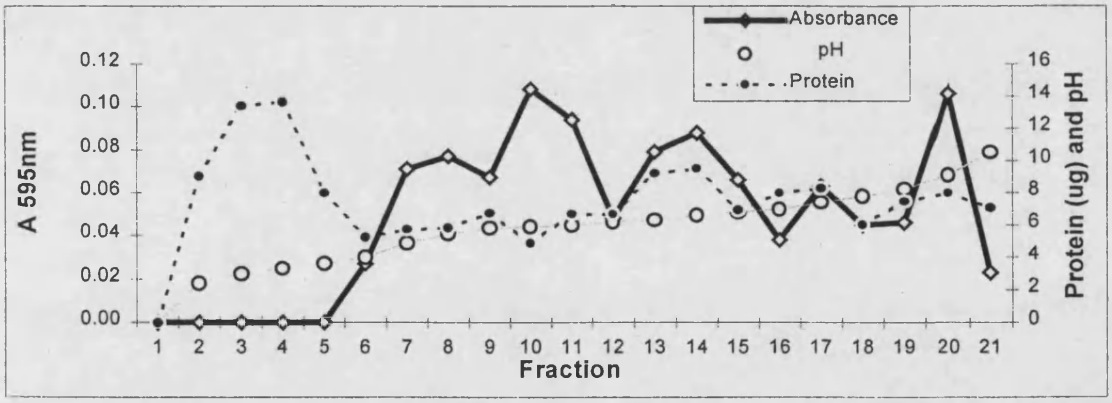
Figure 17 : Zymograms for (a) arabinosidase and (b) xylanase activities on IEF urea gels and using deglycosylated samples treated with urea and β-mercaptoethanol. **1 - 3: IWF** at 6 days of 471, 171 and Law, respectively; **4:** control leaves; **5 - 8:** droplets at 64 hours of 171, 471, Ln97, Law, respectively; **9:** culture filtrate.



(a) First running

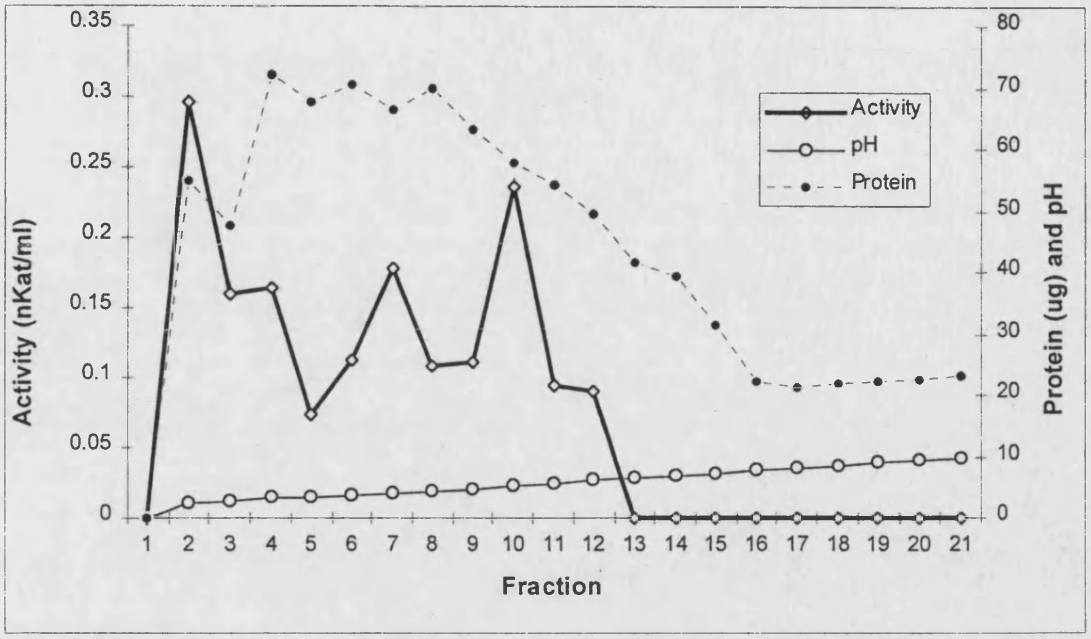


(b) Second running (fraction 6 to 11 of first running) expressed as reducing sugar equivalents

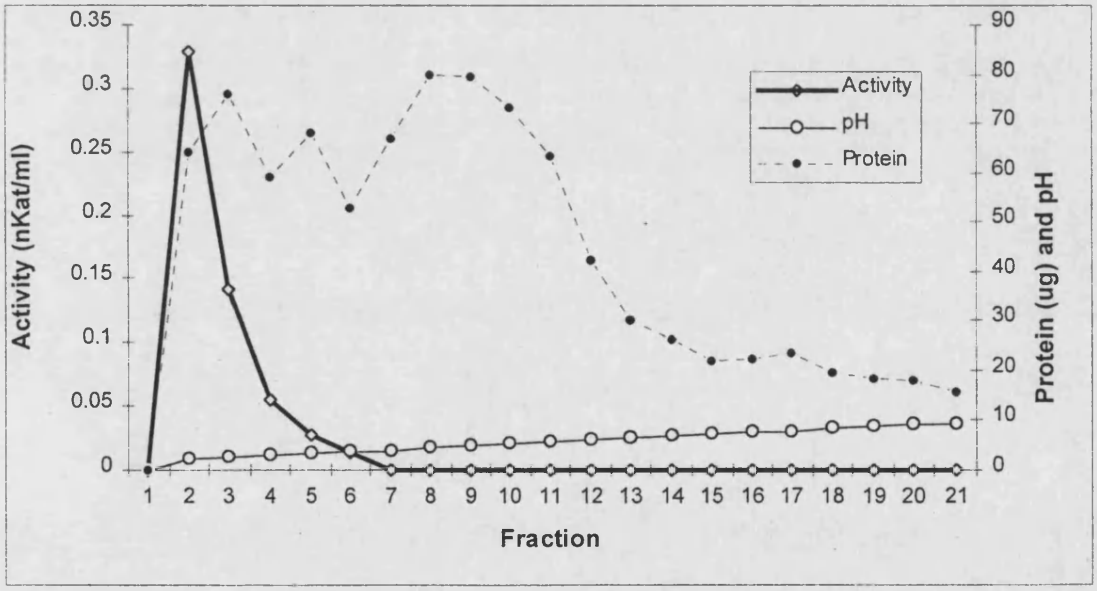


(c) Second running (fraction 6 to 11 of first running) expressed as absorbance from the Remazol Brilliant Blue-Xylan assay

Figure 18 : Rotofor isoelectric focusing patterns of extracellular xylanase released by *S. nodorum* in culture filtrates using different detection methods; (a), first running of liquid culture filtrate expressed as reducing sugar equivalents; (b) second running of liquid culture filtrate with fractions of the first running that showed xylanase activity (6 to11), expressed as reducing sugar equivalents; (c) Same fractions (fraction 6 to 11 of first running) expressed as absorbance at 595 nm (Remazol Brilliant Blue-Xylan assay). One of the two experiments performed is showed.



(a) Treated



(b) Control

Figure 19 : Rotofor isoelectric focusing patterns of xylanase in extracts from (a) leaves infected with *S. nodorum*, and (b) control leaves. Activity is expressed as reducing sugar equivalents.

overlaying and detection techniques. The best focusing was obtained with the use of deglycosylated samples in composite agarose gels and ampholytes in a range of pH 3.0 to 8.0; and overlaying with Remazol Brilliant Blue. However, there was still smearing that made it difficult to identify individual isoforms (Fig 16b).

After improving the conditions for IEF as stated before (see 2.3.5.1), it was possible to resolve two xylanase isoforms (pI's 4.5 and 4.0) as shown in figure 17b. The isoforms were present in the IWF of inoculated plants, in inoculating droplets and also in the culture filtrate, including in a group of 7 to 10 closely spaced bands that differed in isoelectric point by small increments, which was indicating charge microheterogeneity. One xylanase isoform of pI 3.5 was resolved in the control IWF.

2.4 Discussion

Stagonospora nodorum culture supernatants and extracts of inoculated leaves and controls were assayed for different CWDE. Among them, levels of xylanase, arabinosidase and xylosidase were present *in vitro* and in infected tissues always at higher levels than in corresponding controls. The three enzymes showed steadily increasing activity in early stages *in vitro* and *in vivo*. This similar pattern is significant, when comparing the droplets experiment, in which host activities are much less likely to be present, with IWF and leaf extracts.

Comparison of IEF patterns of proteins released by *S. nodorum* in culture filtrates and proteins extracted from inoculated and healthy wheat tissues revealed the presence, in the invaded tissues, of a plant xylanase and some arabinosidases, and several other forms, probably of pathogen origin. Fungal origin is likely because arabinosidase and xylanase isoforms were identified in culture fluids, inoculation droplets, and in the IWF and extracts of inoculated tissues. These forms appeared during the course of infection by three different isolates, as suggested by results from analytical IEF of inoculation droplets and the IWF.

Production of these enzymes *in vitro* by two of the most contrasting isolates, in terms of aggressiveness (size and rate of spreading of lesions), was not significantly different. On the contrary, Lalaouia *et al.* (1999) found that a highly aggressive isolate of *S. nodorum* produced more xylanase, cellulase and polygalacturonase than two weakly aggressive isolates, when *in vitro* production of some CWDE by *S. nodorum* were compared. It is possible that the isolates used for comparison for Lalaouia *et al* were more different in aggressiveness than those used in this study. Alternatively, the link may be merely coincidental as only 3 strains were compared and conditions *in vitro* are unlikely to reproduce closely those during infection.

Nevertheless, in this study, the three different *S. nodorum* isolates did show correlation between aggressiveness and levels of arabinosidase and xylanase activities *in vivo*, e.g. BS 471 (the most aggressive isolate) produced more of both enzymes than BS 171 and LAW did (less aggressive isolates) in IWF of inoculated plants and in inoculation droplets. Also, some physico-chemical characteristics of the culture fluids (as viscosity) and the proteins (carbohydrate content) seemed to differ, the culture filtrate of BS 471 was the most viscous and contained the highest carbohydrate content (Chapter 3). A possible explanation is that differences in aggressiveness among the isolates used in this study are correlated with the degree of glycosylation of the secreted proteins more than with the amount of proteins produced. Then, differences of activities found *in vivo* but not *in vitro* could be accounted for by the stability of the enzymes (e.g. its resistance to host proteases) more than by the quantities of proteins produced by the isolates.

Arabinosidase and xylanase activities appeared before (droplets experiment) and were coincident with symptoms (IWF and extracts of inoculated tissues). It is possible that the differences in the levels of activities between inoculated leaves and controls are accounted by the fungus. If this is the case, degradation of the plant cell wall at early stages of the infection of *S. nodorum* on wheat might be a necessary event for penetration and release of nutrients for fungal growth and colonisation of host tissues (in agreement with results of Karjalainen & Lounatmaa, 1986). Immuno-cytochemical techniques need to be employed to localise these enzymes during the infection process *in situ*.

The expression of these enzymes are likely to reflect the composition of the primary cell wall of grasses in which xylan replaces pectin as the main matrix component, and in accordance, polygalacturonase activity occurred transiently in this extensive study as was the case in previous reports (Carlile, 1999; Lehtinen, 1993; Magro, 1984).

Viscometric assays for endo-xylanase and endo-polygalacturonase reinforced the predominance of xylanase. Xylanase increased steadily in extracts from infected leaves, whilst endo-PG activity in inoculated leaves remained at control levels.

The fungal xylanase appeared to have several isoforms *in vitro*, as it was visualised in large clearing areas, in the acidic pH range. Lehtinen (1993) also found multiple xylanase isoforms in liquid culture filtrates of *S. nodorum* grown on wheat cell walls. The author suggested the possibility that such patterns could be artefacts resulting from ampholyte binding to proteins as was found for cellulases; or from proteolytic activity, giving numerous active oligopeptides (Gianazza, 1995). Another explanation for the behaviour of this enzyme during focusing includes glycosylation and/or aggregation (Imperiali and O'Connor, 1999).

During this study, proteolytic activity was assumed to be prevented with the use of antiprotease cocktail (appendix 2). The samples, especially IWF were ultracentifuged through 50,000 and 10,000 MW cut-off membranes, to remove the likely effects of polysaccharides during running. Nevertheless, both fractions above 10,000 exhibited similar behaviour. The proteins were positive for the Periodic Acid-Schiff reaction, indicating high glycosylation (Chapter 3).

So far, the results of this study indicate that *S. nodorum* possesses several xylanase isoforms, but there is not proof that all peaks in the preparative Rotofor Cell, correspond to real isoforms. Yet, possible aggregation and/or molecular interactions need to be counterbalanced to achieve sufficient resolution revealed by overlay gels. In any event, it is not surprising that diverse isoforms are present in this system since studies on other microbial CWDE have revealed considerable complexity in most cases (Wu *et al.*, 1995; Holden &Walton, 1992).

Factors responsible for the multiplicity of xylanases include differential mRNA processing, postsecretional modification by proteolysis of larger preforms, and post-translational modification such as glycosylation and autoaggregation (Gianazza, 1995; Coughlan *et al.*, 1993; Biely, 1985). Multiple xylanases can also be the product from

different alleles of the same gene (Wong *et al.*, 1988) or be result of independent genes (Hazlewood and Gilbert, 1993). Also, the advantages of multiplicity of forms in CWDE conferring flexibility to a pathogen have been recognised (Keon *et al.*, 1987); such as endo–PGs secreted by *Verticillium albo-atrum* and *Monilinia spp*; endopectate lyases secreted by *Hyphomyces solani* f,sp *cucurbitae* and *Erwinia carotovora* and the multiple xylanases of *Aspergillus niger* (Wong *et al*1988), *Trichoderma viride* (Biely *et al*,. 1985), and *Phanerochaete chrysosporium* (Dobozi *et al.*, 1992).

Studies attempting to correlate CWDE production to virulence or pathogenicity in phytopathogenic fungi have been complicated by multiple isozymes of each CWDE produced by most fungi. Different isozymes of each CWDE may have different functions and may be produced at different stages during infection of plant tissue (Cooper, 1993; Keon *et al.*, 1987)

Because of the heterogeneity of xylan, its hydrolysis requires the action not just of xylanase as a depolymerase but also other accessory enzymes which remove side groups from complex polymers as they are they exist in the plant cell wall. Side groups can constitute a steric impediment for cleaving of adjacent bonds and presumably therefore, glycosidases corresponding to the most important appendages of the polymer would play an important role in its complete degradation (Sunna and Antranikian, 1997). The most common side groups of xylan are acetyl, arabinofuranosyl and glucuronosyl residues (Joselau *et al.*, 1992), and according to Mai *et al.* (2000), complete and rapid degradation of branched and substituted xylan requires primarily the synergistic action of α-arabinosidases, and β-xylosidases. Synergy and enzymatic complexes are treated in more detail in Chapter 3.

β-xylosidases and α-arabinosidases have been described from a variety of microorganisms, including plant pathogens and have been purified both from bacteria and saprophytic fungi.

To the present, only one β-xylosidase has been isolated from a fungal pathogen (Ranson & Walton 1997). The primary sequence of the *Cochliobolus carbonum* Xyp1 was unrelated to any known eukaryotic β-xylosidase but had 35% overall identity to two bacterial bifunctional β-xylosidase/α-arabinosidases. Mutants for gene disruption were produced (Wegener *et al.*, 1999) and although they lost major β-xylosidase activity, were still fully pathogenic on maize.

Only few α-arabinosidases have been purified and characterised from fungal pathogens such as that of *Sclerotinia sclerotiorum* (Baker *et al.*, 1979) and only in two cases have they been implicated as virulence factors. Howell (1975) found correlation of arabinosidase production with pathogenicity of induced mutants of *Monilinia fructigena* and Rehnstrom *et al.* (1994) found that arabinosidase-deficient mutants of *Sclerotinia trifoliorum* have reduced virulence on alfalfa stems.

Arabinosidase facilitates arabinoxylan hydrolysis by xylanase by cleaving arabinoside substituents from the main chain (Nishitani & Nevins, 1991; Cooper *et al.*, 1988). Moreover, many xylanases of fungal origin such as from *Neurospora crassa* and *Aspergillus niger*, were found to release arabinose from arabinoxylan. A novel enzyme has been isolated from *Aspergillus awamori*, which cleaves arabinose substituents from cereal arabinoxylans, and which is specific to the substituent on the relevant xylose of the main chain and does not cleaves the xylan main linkage (Kulkarni *et al.*, 1999). Thus, it may be significant that among all tested glycosidases, arabinosidase showed the highest level in culture filtrates and in infected leaves.

Preparative IEF of the culture filtrates resolved two arabinosidase isoforms. Their pIs are similar to others reported for arabinosidases of fungi and bacteria (Greve *et al.*, 1984; Laborda *et al.*, 1974). Analytical IEF revealed that these isoforms appeared in the inoculation droplets and IWF of inoculated tissues.

The low number of isoenzyme forms exhibited for arabinosidase makes this protein an interesting candidate to study the effect on virulence of single gene disruption, to resolve its likely importance as an accessory enzyme for effective degradation of xylans.

Interestingly, an arabinosidase from *Cochliobolus carbonum* pathogenic to maize has been proposed recently as a candidate for a virulence gene while other single genes for several CWDE have been excluded from making a major contribution to the virulence of *C. carbonum* (Tonukari *et al.*, 2000).

In summary, levels of xylanase and arabinosidase in inoculating droplets, IWF and extracts of inoculated tissues and controls indicated that these activities are probably of pathogen origin. This was supported by comparison of analytical IEF for IWF and extracts of inoculated tissues and controls. Both enzymatic activities were correlated positively with the aggressivity of three different isolates used to inoculate wheat leaves.

At this stage it is tempting to assign possible role(s) for one or more arabinoxylan-degrading enzymes in pathogenicity, based on their predominance *in vitro* and *in planta*, detection at early stages of infection and correlation with development of symptoms and aggresiveness of isolates.

CHAPTER 3 EVIDENCE FOR GLYCOSYLATED ARABINOSIDASE-XYLANASE MULTIENZYMATIC COMPLEX IN *Stagonospora nodorum*

3. 1 Introduction

During this research it became evident that the aberrant properties of *Stagonospora nodorum* arabinosidases and xylanases (see Chapter 2) might be explained by their existence as a glycosylated, multienzymatic complex. Some of the properties of conjugated proteins and complexes and the methods used to study them are outlined below.

3. 1. 1 Glycoproteins and Sialic acids

Proteins contain one or more polypeptide chains, each having one hundred or more α-amino acid residues as building blocks. On the basis of their composition they may be divided into two major classes: simple (non-conjugated) and conjugated proteins. Conjugated proteins contain in addition to the amino acid residues organic and/or inorganic components. Among the different classes of conjugated proteins, glycoproteins are the most biologically significant group. Glycoproteins are widely distributed and include intracellular and, in particular, extracellular proteins. Many enzymes are glycoproteins and in some cases (e.g. some hormones), the carbohydrate part was demonstrated to be essential for biological activity (Durchschlag and Jaenicke, 1997).

Glycoproteins contain carbohydrate groups attached covalently to the polypeptide chain; the percentage by weight of saccharide chains in different glycoproteins may vary,

usually containing not more than 2 to 10 saccharide monosacharides per chain (Durchschlang and Janenicke, 1997; Gianazza, 1995).

Glycans are conjugated to the polypeptide chains by two types of primary covalent linkages, *N*-glycosyl and *O*-glycosyl. The former is called an asparagine-linked sugar chain and contains an *N*-acetylglucosamine (GluNAc) residue that is linked to the amide group of asparagine residues of the polypeptide. The latter contains at its reducing end a *N*-acetylgalactosamine (GalNAc) residue that is linked to the hydroxyl group of either serine or threonine of a polypeptide. This linkage is called an *O*-linked sugar chain or mucin type. Some glycoproteins have both the *N*-linked and *O*-linked forms in their molecules (*N*, *O*-glycoproteins) (Imperiali and O'Connor, 1999; Wyss and Wagner, 1996).

The addition of carbohydrate to a polypeptide chain will change the shape and size of the protein structure and can dramatically alter the structure, and consequently the function, of the polypeptide to which they are attached. Several important discoveries have revealed the following biological roles of glycans: (i) Protection of polypeptide chains against proteolytic enzymes; (ii) influence on heat stability, solubility and many other physico-chemical properties; (iii) Interaction with other proteins or non-protein components of the cell including control of the life time of circulating glycoproteins and ultimately cells (Imperiali and O'Connor, 1999; Kishino and Miyazaki, 1997).

As a rule, the form of protein glycosylation is highly heterogeneous. The variation of carbohydrate primary structure between molecules of a glycoprotein is known as microheterogeneity and can take several forms: the oligosaccharide chains may be linear or branched and can vary also in their length, with variation in the number and type of terminal sialic acid residues, if sialic acid is present (Gianazza, 1995; Beeley, 1985). Microheterogeneity in respect to the carbohydrate moiety was found to be due to the occurrence of di-, tri-, and tetra- glycans of the N-acetylactosamine type at the five glycosylation sites (Kishino and Miyizaki, 1997).

In turn, there are approximately 40 members of the sialic acid family that have been identified so far. Sialic acids are a family of monosaccharides comprising several derivatives of neuraminic acid. In the past decades, sialic acids have been characterised mostly in animal cells, some viruses, bacteria and protozoan. Sialic acids can be found in fungi. They were first demonstrated in yeast like cells of *Sporothrix shenckii* in 1982 (Alviano *et al.*, 1982). Benhamou and Ouellette (1987) reported for the first time sialic acids in phytopathogenic fungus, showing that they were abundant in the cell surface component of *Ascocalix abietina*, where they were thought to influence fungal resistance to unfavourable environmental conditions.

The diversity of sialic acids is generated by different substituents occurring on the particular 5- and 9- carbons, but there may be substitutions present on carbons 4, 7 and 8 as well. Substitutions in carbon 5 defines the four major types of sialic acid: neuraminic acid (Neu), *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and ketodeoxynonulosonic acid (KDN). *O*-acetylation is one of the most common modifications that occur on sialic acids; the role and biological importance of this modification which was first thought to be a "species-specific" determinant, has only started to be demonstrated in last few years, more than 40 years after its discovery. Very little information is available about sialic acid in plant pathogenic fungi (Alviano *et al.*, 1999; Klein and Roussel, 1998).

Due to the low quantities of some modified sialic acids and the insensitivity of earlier techniques, *O*-acetylated sialic acids have been ignored in numerous samples and their widespread distribution has almost certainly been underestimated. The presence of *O*-acetylated sialic acid is indicated by the difference before and after saponification (base treatment) in response of the Warren 2-thiobarbituric acid (TBA) assay for sialic acids. The most usual way to study modified sialic acids is first to release, then purify and quantify them. Sialic acid can be released by acid hydrolysis or enzymatically. Sialidase digestion does not alter the modified sialic acids, however, the specificity of sialidase toward the linkage (2→3, 2→6 or 2→8) to the underlying glycoconjugate and the nature

of the sialic acid explain the partial release generally observed (Manzi and Varki, 1993; Roussel, 1998).

3. 1. 2 Multienzymatic Complexes

Because many CWDE are part of multienzymatic complexes, the nomenclature of such systems is reviewed. According to the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) at (http://www.chem.qmw.ac.uk/ iubmb/misc/menz.html) a multienzyme is a protein possessing more than one catalytic function contributed by distinct parts of a polypeptide chain or by different subunits or both. A multienzyme complex is a multienzyme with catalytic domains on more than one type of polypeptide chain. If the catalytic domains are located in the same polypeptide this is consider as a multienzyme polypetide.

Presence of multienzyme complexes can significantly enhance the metabolic efficiency of a cell. Microorganisms utilising complex substrates with extensive microheterogeineities such as carbohydrates have evolved batteries of multifunctional glycanases, facilitating parsimonious utilisation of these substrates. With multifunctional enzymes regulation of metabolic fluxes is easier; the stability of the labile substrates is enhanced and the diffusion of intermediates can be controlled (Kumar and Deobagkar, 1996). According to these authors multifunctionality in hydrolases includes multienzyme complexes and multifunctional glycanases (multienzyme polypeptides) produced by bacteria and fungi.

Examples of microbial multienzyme complexes include the cellulosome synthesized by *Clostridium thermocellum* (Lammed *et al.*, 1984), the xylanosome synthesized by *Butyrivibrio fibrisolvens* H 17c (Thomsom, 1993) and a cellulase-xylanase-β-glucosidase complex produced by *Trichoderma reesei* (Sprey & Lambert, 1983). Other fungi and bacteria encoding multifunctional glycanases are shown in Table 2.

Table 2. Multifunctional Glucanases (multienzyme polypeptides) (Kumar and Deobagkar, 1996)

Multifunctional Glucanases	
Enzyme	**Organism and Reference**
β-glucosidase / β-xylosidase	*Chaetomium trilaterale* [54]
XylA-a xylanase with 2 identical catalytic domains	*Neocallimastix patriciarum* [69]
XlnA - a xylanase with 2 distinct catalytic domains	*Ruminoccus flavefaciens* [14]
xylanase / xylosidase	*Caldocellum saccharolyticum* [36]
xylosidase / arabinofuranosidase	*Butyrivibrio fibrisolvens* [59], *Clostridium stercorarium* [49], *Bacteroides ovatus* [66], *Trichoderma reesei* [46]
xylanase / arabinofuranosidase	*Fibrobacter succinogenes* [37]
cellulase / xylanase	*Irpex lacteus* [33], *Trichoderma viride* [55]
cellulase with exo- and endo- activities	*Caldocellum saccharolyticum* [51]
cellulase / β-(1,3)-glucanase	*Ruminoccus flavefaciens* [15]
cellulase / lichenase / xylanase	*Clostridium thermocellum* [20], *Cellulomonas fimi* [7]
amylase with α- and β- activities	*Bacillus polymyxa* [58]
amylases with α-amylase and pullulanase activities	various thermophilic bacteria [35,48]
glucoamylase / pullulanase	*Hormoconis resinae* [13]
amylase / maltase / cyclodextrinase	*Bacillus subtilis* [27]

3. 1. 3 Unfolded State for Studying Properties of Complex Proteins

According to Creighton (1993; 1979), the random coil might be considered the natural state of a polymer, in which the multiple interactions that occur simultaneously in any particular conformation would be expected to stabilise each other. Many unfolded proteins in strong denaturants such as 6 M guanidium chloride or 6–8 M urea or at extremes pH, have been shown to have the average hydrodynamic, physical and thermodynamic properties expected of random-coil polypeptides.

The precise physical explanation of the denaturing ability of compounds like urea or guanidium chloride is not clear, but studies with model compounds suggest that both function by increasing the aqueous solubility of the hydrophobic portions of proteins while maintaining the hydrogen-binding capability of the aqueous solvent. The unfolding of proteins by these reagents has generally been studied experimentally by measuring one or more physical parameters of the protein as a function of the concentration of the reagent. One physical property in which folded and unfolded proteins differ markedly is the shape and size of the molecular volume occupied by the polypeptide, unfolded proteins being remarkably compact relative to the folded polypeptide (Creighton, 1996).

The shape of a protein affects the rate at which it migrates through polyacrylamide gels and the rate of electrophoretic migration through such gels has been shown to be sensitive to the compactness of intermediates during protein folding. Thus, if electrophoresis is carried out in a gradient concentration of urea, perpendicular to the direction of migration of a protein, the folding transitions can be visualised in the polyacrylamide slab. This is because molecules at varying positions along the gel can migrate at different speeds in varying concentrations of urea. It has been demonstrated that this approach is useful in

gaining information about urea-induced transitions of proteins, which is not possible to obtain with other techniques (Creighton, 1979; Gianazza *et al.*, 1997; Kresl *et al.*, 1998).

3. 2 Materials and Methods

3. 2. 1 Biological Material

Stagonospora nodorum isolates BS 171 and BS 471 were maintained as described in Chapter 2 (section 2.2.1). Wheat plants, variety Riband, were grown as described in 2.2.2 and cell walls were obtained as described in 2.2.2.1.

3. 2. 2 Preparation of *S. nodorum* Liquid Culture for Production of Soluble and Cell Wall Adsorbed Proteins.

S. nodorum liquid cultures were set up as described in section 2.2.4 for 72 h. Following this culture fluids and pellet were obtained by centrifugation at 10,000 x g for 10 min. Supernatants were filtered successively through 5 μm, 1 μm and finally 0.44 μm membranes to obtain clear culture filtrates. These solutions were kept on ice for further protein fractionation.

The pelleted cell wall and mycelia were washed extensively; first with sterile distilled water and then with sterile 25 mM phosphate buffer pH 6.0. The drained pellet was then suspended in two volumes of the same buffer plus 0.5 M NaCl to desorb ionically bound proteins from the cell walls. The suspension was agitated for 30 min at room temperature and then centrifuged at 10,000 x g for 10 min. Supernatant containing the desorbed proteins was dialysed for 12 h with two changes, vs 10 mM Tris-HCl buffer pH 6.0.

Finally, the protein solution was concentrated by laying the dialysis tubing on polyethyleneglycol beds (section 2.2.3.1). Concentrated samples were kept at 4 °C for analysis.

3. 2. 3 Bulk Protein Precipitation and Ammonium Sulphate Fractionation for Large Volumes of Protein Solution

Culture filtrates were chilled to 0 °C and total protein precipitation was carried out with stepwise addition of high purity ammonium sulphate (DBH) up to 85% (w/v) saturation at 0 °C (559 g to a litre of solution). The mixture was allowed to equilibrate overnight before centrifugation at 12000xg for 10 min in a precooled centrifuge at 0 °C. Supernatants were drawn off by suction and the precipitate used for fractionation.

A solution of 3.9 M ammonium sulphate was made at 0°C and then adjusted to pH 7 with NH_4OH. From this, a subset of solutions were prepared giving 65%, 55%, 45%, 35% and 15% (w/v); each of them was used in a volume of 1/ 10 that of the original volume from which the bulk precipitate was made. Starting from the highest concentration, each extracting solution was added to the pelleted protein, and the mixture stirred for 30 min on ice slurry for equilibration. After each extraction, the mixture was centrifuged as described and the supernatants decanted and kept. A final extraction was made for residual protein and kept for analysis. All saved materials were dialysed, concentrated on polyethyleneglycol beds and assayed for protein content and for xylanase and arabinosidase activities.

3. 2. 4 Alternative Methods for Concentrating Medium or Small Volumes of Protein Solutions

When it was necessary (e.g. for electrophoresis, loading chromatography columns), medium or small volumes of protein solutions were concentrated by osmosis, acetone precipitation or lyophilization.

Usually after dialysis, samples were concentrated by placing the dialysis tubing on polyethyleneglycol beds (PEG, 35'000, Fluka) and allowing the solvent to pass through the membrane by osmotic pressure, until a smaller volume was obtained. The tubing was then washed extensively under tap water to remove the polyethyleneglycol and the sample pipetted out. For organic solvent precipitation, HPLC grade acetone (ACS Reagent, Sigma), kept at -20 °C was brought into the lab on dry ice and was added dropwise to the protein solution, in the ratio of two volumes of organic solvent per one volume of the solution. For lyophilization, up to 400 µl protein solution was pipetted into 1.5 ml Eppendorf tube and fast-frozen by immersion in liquid nitrogen. Frozen samples were then lyophilized in a precooled (-40 °C) speed vac concentrator (Savant Speed Vac Concentrator, Stratatech Scientific).

3. 2. 5 Determination of Protein Concentration and Enzyme Assays

The protein content of each sample was determined colorimetrically using the protocol of Stoscheck (1990) as described in section 2.2.5. Arabinosidase, and xylosidase activities were measured as described in section 2.2.5.4. All xylanase activities described in this chapter were measured using the RBB-xylan dye protocol, as stated in 2.2.5.3.

3. 2. 6 Ion Exchange Chromatography

Ion exchange chromatography in columns was attempted as a first purification step for xylanases and/or arabinofuranosidases. Batch procedures were also used to remove putative charged impurities from samples. Cation exchange suports used were SP Sepharose Fast Flow (Pharmacia) and AG MP-50 (hydrogen form, BioRad); anion exchange resins employed were Q Sepharose Fast Flow (Pharmacia) and AG MP-1 (chloride form, BioRad)

3. 2. 6. 1 Ion Exchange in Columns

Protein separation was carried out using 25 ml of wet, fully regenerated resins loaded into a glass column (16 x 140 mm) which was then equilibrated with extensive washing in the respective buffer at a flow rate of 1ml/min. All chromatography was performed at room temperature

Buffers used were: Tricine 10 mM pH 9.0 for the strong cation exchanger. Tricine 25 mM pH 8.0, Succinate 25 mM pH 5.6 and pH 5.0 and Acetate 20 mM pH 4.5 for the weak cation exchanger. Tris-maleate 25 mM pH 6.0 and Sodium-phosphate 10 mM pH 7.0 for the weak anion exchanger. Finally, Tricine 10 mM pH 8.0 was used for the strong anion exchanger.

Concentrated protein sample (liquid filtrate or ammonium sulphate fractionation samples) containing 6 to 10 mg total protein were desalted and equilibrated with running buffer by gel filtration. Samples (2 ml) were then applied to the ion exchange column and unbound proteins eluted using a flow rate of 1.0 ml/min for 60 min. Elution of bound proteins was effected by linear gradient of 60 ml of elution buffer and 60 ml of buffer containing 1 M NaCl. Fractions of the eluate (2ml) were monitored for protein content by reading A 205 nm. Chromatograms were constructed and peak fractions pooled together and dialysed against buffer Tris-HCl 10 mM pH 6.0 for 12 h with one change. Peaks were later

concentrated on polyethylenglycol beds and analysed for xylanase and arabinofuranosidase content. In relevant fractions, the protein content was determined colorimetrically.

3. 2. 6. 2 Ion Exchange in Batch

This technique was employed to remove small charged molecules in the protein samples that could be interfering with electrophoresis or enzymatic activity. Removal of putative charged carbohydrates and/or ions was done with the mixed bed resin AG 501-X8 (20-50 mesh, BioRad) and Amberlite IRA-400 (chloride form, Sigma). Removal of sodium dodecyl sulphate (SDS) from samples to allow renaturation of the proteins and recovery of enzymatic activity was performed with the anion exchange resin AG 1-X8 (200-400 mesh, chloride from BioRad).

The ion exchange resins were exhaustively washed in a Buchner funnel with the following solvents: 2N NaOH, distilled water, 4N acetic acid, distilled water, and finally 20m M Tris-HCl buffer, pH 6.0. The resins were stored in this solution for several weeks at room temperature.

For removal of suspected charged molecules contaminating the protein solution, a given volume of sample was mixed with half that volume of ion exchange resin slurry and allowed to equilibrate for 10 min with agitation. The resin was removed by passing the suspension through a 2-ml syringe equipped with a very fine needle. Application of gentle pressure with the plunger allowed the solution to pass through the needle while the resin was retained.

For SDS removal, 50 to 100 µl of the thick anion exchanger slurry was added to fractions with SDS, incubated at room temperature for 5 min with agitation in a CM-9 shaker (Sarstedt), allowing the SDS to bind to the support. Finally, tubes were centrifuged at

2000 rpm for 5 min on a bench centrifuge and the supernatant containing renatured proteins pipetted out in new Eppendorf tubes.

3. 2. 7 Affinity Chromatography

Affinity chromatography was the used as a method of separation to achieve purification of arabinofuranosidase and xylanase in few steps (and consequently in high yields). The approach used in this study was the crosslinking of natural substrates by the bifunctional agent epichlorohydrin, which have been used successfully in the purification of several polysaccharide-splitting enzymes as well as some glycosidases. Also, the widely used lectin Concavalin A was assessed as a possible affinity ligand for the mentioned enzymes.

3. 2. 7. 1 Preliminary Assessment of Xylan and Concanavalin A as Affinity ligands for Xylanases and/or Arabinofuranosidases

Culture filtrate aliquots containing 150μg of protein were mixed with 50 mg of each oat spelts xylan (Sigma) and Concanavalin A (Con A-Sepharose, Sigma); suspended in 1 ml of 50 mM Tris-HCl buffer pH 7.0 in 1.5 ml Eppendorf tubes. The suspensions were agitated for 30 min at room temperature on a CM-9 shaker (Sarstedt) and later centrifuged at 10,000 rpm for 5 min on an Eppendorf 5415C bench centrifuge. Supernatants were pipetted out and the pellet washed once with 1 ml of the same buffer.

After centrifugation and decanting, proteins were desorbed from substrates by mixing them with 500 μl of the respective buffer plus 1 M NaCl for xylan, and 0.25 M manopyranoside and 0.25 M glucopyranoside for Con-A. Suspensions were left to equilibrate for 30 min under agitation at room temperature and later centrifuged as mentioned above.

Finally, first and last supernatants were dialysed for 12 h with two changes *vs* 50 mM acetate buffer pH 5.5, concentrated on polyethylenglycol beds to 200 µl and assayed for xylanase activity. The degree of adsorption of the xylanase activity was determined by taking the difference between total enzyme activity of controls (without ligands) and activity of both supernatants.

3. 2. 7. 2 Preparation of Cross-Linked Xylans

The procedure described by Rozie *et al.* (1992), on the purification of xylanases from *Aspergillus niger*, was followed. Xylans used to prepare crosslinked adsorbents were from oat spelts (Sigma) and birchwood (Sigma).

In a fume extraction cabinet, 8.88 g of each of the above described xylans was suspended in 12.52 ml of distilled water and 26.54 ml of 5M NaOH (AnalaR grade, BDH), in 100-ml beakers. The suspension was stirred with a magnetic stirrer and heated up to 40 °C.When it became too viscous, it was necessary to continue homogenising with a spatula. Then 10.55 ml (+)-Epichlorohydrin (Aldrich) was added. Homogenisation continued until the mixture gelatinised after 15-30 min, when stirring was no longer possible. Reaction was continued for 24 h at 40 °C.

Next, the temperature was increased up to 70 °C for about 16 h, when the gelatinised compound appeared dry. The gel was then crushed with pestle and mortar until fine particles were obtained. The fine particles were transferred to a 1-litre flask and about 1 l of 7% (w/w) acetic acid was added. The mixture was stirred thoroughly and then left to sediment. After decanting, the precipitate was washed three times with 1 litre of water. Finally, a powder was isolated by lyophilization of the slurry. The yields of the dry crosslinked xylans were 95% (oat spelts) and 90% (birchwood).

3. 2. 7. 3 Preparation of Cross-Linked Arabinans

Preparation of crosslinked arabinans were carried out as described by Waibel *et al.* (1980) using commercial arabinan (from sugar beet, Megazyme) and arabinan previously purified in our laboratory also from sugar beet (lab-arabinan).

2.5 ml epichlorohydrin and 10ml 30% (w/v) NaOH were added in 100-ml beakers in a fume cabinet with vigorous stirring to 5 g of each arabinan. Each reaction was stirred at room temperature, until it was not possible because of gelatinisation. The reaction continued for 4 h at the end of which the gel appeared dry. This was then crushed with pestle and mortar until fine powder was obtained. The powder was later neutralised with 75 ml of 7% (w/w) acetic acid, followed by three washes of distilled water. The gelled crosslinked arabinan was finally filtered, rinsed with 70% (w/w) ethanol and freeze-dried. The weights of the dryed crosslinked arabinans were 4.8g (for the commercial) and 4.5 g (lab-arabinan), which are around 90% of yield.

3. 2. 7. 4 Influence of pH on the Adsorption of Proteins by the Cross-Linked Arabinans and Xylans

A set of buffers (20 mM) in the pH range of 3.7 to 8.0 were prepared as follows: Sodium-acetate buffers pH 3.7, 4.0, 4.6, 5.4 and Sodium-phosphate buffers pH 6.2, 7.0 and 8.0. Then, in 1.5 ml Eppendorf tubes, 100 mg of crosslinked arabinans and xylans were suspended in 1 ml of either sodium-acetate or sodium-phosphate buffers at each mentioned pH. Suspensions were incubated at room temperature for 30 min with constant agitation on a CM-9 shaker (Sarstedt), allowing equilibration.

Next, the tubes were centrifuged at 2,000 rpm for 5 min in an Eppendorf 5415C bench centrifuge. Supernatants were discarded and replaced with fresh solutions made up by mixing aliquots of 100 µl of concentrate liquid filtrate (containing 300 µg of protein) and

900 µl of the buffer at the respective pH. Suspensions were incubated again, centrifuged and decanted as mentioned above.

Proteins were desorbed from crosslinked substrates by mixing with 1 ml of the same buffer plus 0.5 M NaCl. Suspensions were left to equilibrate for further 30 min and centrifuge under same conditions. Finally, the supernatants were dialysed for 12 h with two changes vs 50 mM acetate buffer pH 5.5, concentrated on polyethyleneglycol beds to 200 µl and assayed for xylanase and arabinofuranosidase activities. The degree of adsorption of the xylanase and/or arabinofuranosidase activities was determined by taking the difference between enzyme activity of controls (without adsorbents) and activity of the last supernatants.

3. 2. 7. 5 Chromatography on Cross-Linked Xylan and Cross-Linked Arabinan

After electrophoretic analysis of adsorbed proteins in experiment 3.2.6.4., one crosslinked arabinan and one crosslinked xylan were chosen as supports for column chromatography, which was carried out using the model ES-1 Econo System Controller (BioRad).

For this, a hydrated volume of 37 ml of crosslinked birchwood xylan was loaded into a glass column (25 x 70 mm) and equilibrated with 20 mM sodium acetate buffer pH 4.0. Up to 25 ml of liquid filtrate (0.3 µg /µl protein; 7.5 mg total) were chromatographed using a flow rate of 1.0 ml/min, so that the time taken by the sample to go through the support was around 30 min. This parameter is important since it has been demonstrated that this is the optimum time for adsortion of xylanases on crosslinked xylans (Rozie *et al.*, 1992).

After 90 min, the buffer applied was replaced by 20 mM sodium-acetate buffer pH 4.0 containing 500mM NaCl. This buffer was applied over 150 min to desorb all components that were bound to the support. Chromatogram peaks were dialysed against Tris-HCl buffer 10 mM pH 6 for 12 h with one change; concentrated on polyethyleneglycol beds

and analysed for xylanase and arabinofuranosidase activities. In relevant fractions the protein content was determined. After use, the column was regenerated by washing extensively with buffer without NaCl until conductivity returned to basal level.

Similarly, a hydrate volume of 33ml of crosslinked lab-arabinanan was loaded in a glass column (25 x 70 mm) and equilibrated with buffer 20 mM sodium-phosphate buffer pH 8.0. Up to 25 ml of liquid filtrate (0.3 μg /μl protein; 7.5 mg total) was chromatographed using a flow rate of 1.0 ml/min, so that the time taken for the sample to go through the support was around 30 min.

After 90 min, the buffer applied was replaced by 20 mM sodium-phosphate buffer pH 8.0 containing 500 mM NaCl. This buffer was applied for 150 min to desorb all components that were bound to the support. Chromatogram peaks were dialysed against Tris-HCl buffer 10 mM pH 6 for 12 h with one change, concentrated on polyethyleneglycol beds and analysed for xylanase and arabinofuranosidase content. In relevant fractions the protein content was determined. After use the column was regenerated by washing extensively with buffer without NaCl until conductivity returned to basal level.

3. 2. 8 Gel Filtration

This method of separation was used for desalting and cleaning of samples (e.g. removal of suspected oligosaccharides adsorbing to the proteins). Also it was used as a step to purify arabinosidase and xylanase activities. In the first case, desalting was performed by using commercially available, prepack desalting columns (Pharmacia PD10) following the manufacturer's instructions. For cleaning of putative oligosaccharides, protein samples were concentrated by lyophilization. Lyophilized samples were dissolved in denaturing buffer 50 mM Tris-HCl pH 6.8 containing 1% (w/v) sodium dodecyl sulphate (SDS AnalR, BDH) and 280 mM 2-mercaptoethanol. Then, removing of putative tightly bound oligosaccharides was performed as follows.

3. 2. 8. 1 Complete Denatured Samples

When complete denaturation was desirable, a similar method to that described by Paice *et al.* (1978) was followed. Dissolved samples (purified or crude extracts) were boiled for 5 min to unfold completely the protein structure and allow any sugars to desorb. The denatured sample was passed through a Sephadex G-50 column (10 x 50 mm) made from a 5-ml syringe and glass fibre, and equilibrated with 50 mM Tris-HCl buffer pH 6.8, 1% SDS, and 0.04 M 2-mercaptoethanol to separate the proteins from the sugars. Fractions were concentrated again by lyophilization and electrophoresed under denaturing conditions (SDS PAGE), but omitting SDS in the loading buffer.

3. 2. 8. 2 Mild-Denatured Samples

When it was convenient to conserve the enzymatic activity of samples, reverse denaturation was chosen using a similar procedure given by of Weber and Kuter (1971). Lyophilised samples dissolved in denaturing buffer, were incubated for 1 h at room temperature or for 5 to 10 min up to 60 °C (increased temperature irreversibly denatures the enzymes). Under these conditions, dissociation is complete (Reynols and Tanford, 1970). Next, the samples were chromatographed through the same Sephadex G-50 column (10 x 50 mm) equilibrated with 50 mM Tris-HCl buffer pH 6.8 containing 1% SDS and 0.04 M 2-mercaptoethanol. SDS was removed from fractions by a batch procedure, by addition of an anion exchange resin aliquot, as explained in 3.2.6.2.

3. 2. 8. 3 Gel Filtration Chromatography as Purification Step

Two batches of Sephacryl S-100 HR (Pharmacia) were suspended in elution buffer composed of either 20 mM Tris-HCl pH 6.0 plus 0.2M NaCl or buffer 20 mM Tris-HCl pH 6.0 containing 0.2M NaCl, 0.1% (w/v) SDS, 1mM 2-mercaptoethanol. Two columns of 20 x 900 mm were filled with the prepared support (16 x 880 mm bed size with 35 ml

void volume) and equilibrated with the same buffer at 0.25 ml/min flow rate. The columns were standardised with the following purified proteins (all from Sigma): Bovine Serum Albumin (66000 KDa), Egg Albumin (43000 KDa), Carbonic Anhydrase (29000 KDa), Lysozyme (14000 KDa) and Cytochrome c (12400 KDa). With them standard curves were constructed for both columns, plotting the elution volumes *vs* log of molecular weigh (appendix 14).

Proteins for standardisation (10 μg each, 50μg total) or protein samples (250 μg minimum to 500 μg maximum) were dissolved in loading buffer (50 to 100μl maximum) composed of either 20 mM Tris-HCl pH 6.0 plus 0.2M NaCl or buffer 20 mM Tris-HCl pH 6.0 containing 0.2M NaCl, 1% (w/v) SDS, 280mM 2-mercaptoethanol. In the second case, the loading sample was heated up to 60 °C for 5 to 10 min allowing mild denaturation. Samples were laid on the bed and chromatographed at 0.25 ml/min with elution buffer. Eluant was collected in 2ml fractions after 35 ml of void volume and until 150 ml had pass through, when the 2-mercaptoethanol peak was visible. Fractions were monitored at 205 nm for protein content and when it was present, SDS was removed from the fractions as stated in 3.2.6.2. Then, chromatograms were made; protein peaks pooled, dialysed *vs* 10mM Tris-HCl buffer pH 6.0 to remove sodium chloride and concentrated on polyethyleneglycol beds. Finally, xylanase and arabinofuranosidase activities in the pooled peaks were measured and in relevant samples the protein content was determined colorimetrically.

3. 2. 9 Hydroxylapatite Chromatography

Hydroxylapatite chromatography was tested as a technique for separating arabinosidase from xylanase activity. A procedure similar to that described by Gorbunoff (1980) for the purification of ovomucoid was followed, using the model ES-1 Econo System Controller (BioRad).

One part of Bio-Gel HTP (BioRad) was added to six parts starting buffer (sodium-phosphate 1 mM pH 6.8). After decanting the cloudy upper layer and the finest particles at the top of the settled bed, the resin was mixed with an equal volume of buffer and packed in a column of 10 x 200 mm bed volume. The column was equilibrated with 100 ml of the same buffer at 0.5 ml/min flow rate, followed by 100 ml of 1mM NaCl solution at the same flow. With this solution UV and conductivity detectors (Econo system, Bio-Rad) were zeroed. Then, a 1 ml sample containing 0.4 mg protein were concentrated by acetone precipitation, washed with 70% (v/v) ethanol, dissolved in 0.5 ml 1mM NaCl solution and layered on the column.

The column was washed with 1mM NaCl until the eluant showed no UV absorption. This was followed by the addition of 1 mM and then 10 mM sodium-phosphate buffer pH 6.8 to elute adsorbed proteins, then another two steps of 0.5 M NaCl in 10 mM sodium-phosphate buffer pH 6.8 and 0.5 M NaCl solution, which were used to remove the remained proteins from the column.

3.2.10 Electrophoretic Techniques

Analytical IEF and electrophoresis on self-prepared polyacrylamide gels were used during the purification of xylanase and arabinofuranosidase in order to check the purity of proteins and also to find out properties and characteristics of the molecules by using specific additives and staining techniques. Polyacrylamide gels, polyacrylamide-gradient and urea-gradient gels for electrophoresis were prepared for mild-denatured and complete-denatured proteins. Discontinuous buffer systems of Laemmli (1970) were always employed.

3. 2. 10. 1 Isoelectricfocusing and Zymograms

In general, all the techniques described in section 2.2.6 were used. Preparative IEF as a purification step with the Rotofor® system was performed as in 2.2.6.1, using the ammonium sulphate fractions of the culture filtrates as starting material. Analytical IEF, preparing of gels, running of samples, detecting of enzyme activities (zymograms) and determining isoelectric points were accomplished by using the techniques described in section 2.2.6.2.

Zymograms were also used when mild-denatured proteins were electrophoresed. In this case, acrylamide gel was washed before being overlaid, with renaturing buffer for 40 min and with four changes (40 ml each). Renaturing buffer was of composed of 100 mM sodium acetate pH 5.5, 2% Triton X-100 or 100mM sodium acetate pH 5.5 and 8 mM DTT. Detection of electrophoresed proteins under SDS-PAGE conditions was accomplished by fixation and staining of the protein bands (section 3.2.11).

3. 2. 10. 2 Polyacrylamide Gels for Mild-Denatured and Complete Denatured Proteins

Resolving polyacrylamide gel was prepared and cast first. It was composed of 0.375 M Tris- HCl buffer pH 8.8, acrylamide-Bis T10 – C3.9 and 0.1%(w/v) SDS. The stacking gel was prepared when the resolving gel polymerised completely, and was composed of 0.125 M Tris-HCl pH6.8, acrylamide-bis T4 - C3.9, and 0.1% (w/v) SDS. Polymerisation was initiated by the addition of ammonium persulphate (APS, electrophoresis grade, Sigma) at 0.1% final concentration and *N,N,N',N'*-tetraethylmethylendiamine (TEMED, Sigma) at 0.05% final concentration for both gels. To make up the casting solution, the components were prepared as shown in appendix 7B and were mixed in the proportion and in the order shown in appendix 8A. The quantities shown are for dimensions of gels for the Bio-Rad modular Mini-Protean system, using spacers of 0.75 mm.

The casting apparatus was assembled and the resolving gel volume required to leave 1 cm stacking gel (measured from the top of the resolving gel to the bottom of the wells) determined. Monomer solution for appropriate resolving gel was prepared, degassed under vacuum for about 10 min and then the catalyst added. Immediately, the monomer solution was carefully overlayaid with water and the gel allowed to polymerise for 2 h. Then, the top of the resolving gel was rinsed with MilliQ water and the area above it dried with filter paper. Finally, stacking gel was prepared and poured and the comb applied on top of the casting mould.

Sample buffer was composed of 0.06 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.3 M dithiotreitol (DTT, Sigma) and 0.01% (v/v) Bromophenol blue. It was prepared as shown in appendix 7B. One volume of sample (1 to 10 µl) was mixed with two volumes of loading buffer. Samples included a mixture of marker proteins (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad), that were run along side to calibrate the gels.

For complete denaturation, the mixtures of loading buffer and protein samples were brought to 100 °C for 5 min. For mild denaturation the mixtures were incubated at 60°C for about 10 min. After cooling to room temperature, samples were laid on the wells and run at a constant amperage of 10 mA for the stacking gel and 6 mA for the resolving gel, until dye reached the bottom of the resolving gel (usually around 3 h)

3. 2. 10. 3 Polyacrylamide Gradient Gels for Mild-Denatured and Complete Denatured Proteins

A linear polyacrylamide concentration gradient (T = 3.97 to 14.97%) was generated by mixing equal volumes of dense and light solutions of acrylamide in a gradient former (model 385 Bio-Rad). Gel casting solutions were prepared by mixing the components in the proportions and in the order shown in appendix 8B. The quantities shown are for

dimensions of gels for the Bio-Rad modular Mini-Protean system, using spacers of 0.75 mm.

The gradient mixer was positioned on a magnetic stirrer and connected through thin tubing to a peristaltic pump (Econo-Pump model EP-1, Bio-Rad) and casting cassette. After deaerating the light and dense solutions, the reservoir chamber was filled with the light solution and the mixing chamber (containing a stirrer bar) with the dense solution. Next, stirring was started and appropriate volumes of TEMED and ammonium persulphate added to each chamber. The valve between the two chambers was opened and the pump started at a flow rate of 2ml/min; allowing the gradient to form and casting the glass cassette. Immediately, the monomer solution was carefully overlayed with water and the gel allowed to polymerise for 2 h. Then, the top of the resolving gel was rinsed with MilliQ water and the area above it dried with filter paper. Finally, stacking gel was prepared and poured and the comb applied on top of the casting mould. Treatment of samples, loading, and running conditions were as described in section 3.2.10.2 for denatured or mild-denatured proteins.

3.2.10.4 Polyacrylamide and Polyacrylamide Gradient Gels Incuding Xylan for *In Situ* Detection of Xylanase Activity

Xylanase and arabinosidase activities were also detected *in situ* by designing a technique similar to that described by Schuartz *et al.* (1987). Birchwood xylan was included in the resolving gel of acrylamide or acrylamide gradient gels, at a concentration of 0.1% (w/v), as is shown in appendix 9A. Stacking gel was the same as described previously.

Mild-denatured samples were applied and the gel was run at 10 mA, 90 to 100 Volts until the dye reached the bottom of the slab gel. Upon completion of electrophoresis, the gel was washed for 40 min with four changes with cold sodium acetate buffer 0.1 M pH 5.5 containing 8 mM DTT. The gel was then immersed in the same buffer plus 1mM 4-methylumbelliferyl α-L-arabinofuranoside, and incubated at 45°C. After 5 to 10 min the

gel was removed and illuminated under UV at 340 nm to detect activity bands and photographed. The gel was incubated for another 15 min at 45°C. After this, the gel was stained in Coomassie Blue, destained and neutralised by washing with excess of 0.1 M Tris-HCl buffer pH 8.0. Xylanase activity was visualised by staining the gel with Congo red (1 mg/ml) for 10 min at room temperature and destaining in 1M NaCl until yellowish activity bands and blue protein bands were visible on a deep red background.

3.2.10.5 Polyacrylamide Urea-Gradient Gels

Following the electrophoretic analysis of protein unfolding by urea (Creighton, 1979), gels with urea gradients were prepared using the Bio-Rad modular Mini-Protean system plates with spacers of 0.75 mm. The gels were run on the LKB Bromma 2117 Multiphor horizontal electrophoresis apparatus under a constant temperature of 10°C. In order to prepare a gradient of urea concentration perpendicular to the direction of electrophoresis, modifications of the casting system were effected and preparation of the gels set up and standardise as follows:

A U-shape frame was made on one of the glass plates of the Mini-Protean system, by placing three spacers of 0.75mm along the bottom, upper and right side of the rectangular plate. The U-frame had the area corresponding to a resolving gel and was positioned in a way that there was a space of 1 cm, between the upper border of the glass plate and the spacer forming the upper arm of the U-frame. The other glass plate was then clamped together and the cassette put right side down, so the cassette could be filled by the left side (open side of the U-frame).

Then, a linear concentration gradient of urea (0 to 6 M) was generated by mixing equal volumes of dense (6 M urea) and light (no urea) solutions in a gradient former (model 385 Bio-Rad). Gel casting solutions were prepared by mixing the components in the proportions and in the order shown in appendix 9B.

The gradient mixer was position on a magnetic stirrer and connected through a thin tubing to a peristaltic pump (Econo-Pump model EP-1, Bio-Rad) and casting cassette. After deaerating the light and dense solutions for about 5 min, appropriate volumes of TEMED and ammonium persulphate were added to each solution. Immediately, 1.5 ml of the light solution was poured into the reservoir chamber and 1.5 ml of the dense solution poured into the mixing chamber (containing a stirrer bar).

Then, before starting the gradient, 0.5 ml of the dense solution (6 M urea) was fed into the bottom of the cassette (the right side of the plate) giving around 1 cm gel height. The linear gradient followed and when stirring was started, the valve between the two chambers was open and the pump ran at a flow rate of 2ml/min. When the 3-ml gradient finished, 0.5 ml of the light acrylamide solution (without urea) was laid on top, giving another 1 cm height .The gel was left to polymerise for 90 min.

When polymerisation was completed, the clamps were taken out and the spacers forming the two long sides of the u-frame were carefully removed without casting off the cassette. The cassette was turned 90 degrees so that the rectangle had the shorter sides on the left (solution without urea) and on the right (with solution 6M urea). One spacer was positioned on the left side and the space in the upper side filled with the stacking gel solution (approximately 1 cm gel height), without comb.

The stacking gel was left to polymerise for 45 min, after which one of the plates of the cassette was removed and the other left as a support. The cooling system was then set up at 10 °C and the horizontal electrophoresis apparatus prepared. For that, the lateral buffer chambers of the LKB apparatus were filled with the electrode buffer and filter paper wicks were cut at the size of the gel. The gel was then position on the cooling plate using insulating liquid beneath. Then, the wicks were wetted in electrode buffer and applied at the upper and bottom sides of the gel.

Finally, 50μg of purified protein in 50 to 80 μl of loading buffer was layered onto the top of the stacking gel in a continuous line. The loading buffer was composed of 0.06 M Tris-HCl buffer pH 6.8, 10% (v/v) glycerol and traces of bromophenol blue. The gel was run at 900 volts until bromophenol blue reached the end of the gel (2 h 30 min). Then the run was continued for another 2 h 30 min. The lid was removed and a second sample was applied, consisting of 50μg of purified protein mixed with 50 to 80 μl of loading buffer plus SDS 1% and 280mM 2-mercaptoethanol, heated up to 60 °C for 5 to 10 min. After application of the second sample, running was continued for a further 2h 30 min at 900 volts and 8 mA. Total running time for the first protein band was 7h 30 min.

Once the run has finished, the gel was removed from the plate and washed with renaturing buffer 100mM sodium acetate pH 5.5 and 8 mM DTT before being overlayed. Zymograms were described previously.

3.2.10.6 Electroblotting

Upon completion of electrophoresis, gels were soaked for 5 min in electroblotting buffer, composed of 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid buffer, Sigma] pH 11 and 10% (v/v) methanol. A ProBlott membrane (advanced PVDF, Applied Biosystems) was cut to the size of the gel and wetted with methanol for a few sec. Then it was equilibrated in the electroblotting buffer for 5 min. The transblotting sandwich (BioRad mini-Protean System) was assembled according to manufacture's instructions and transfered to electroblott buffer at a constant voltage of 50 volts (170mA-100mA), at room tempararure for 30 min. The ProBlott membrane was removed from the sandwich and rinsed with distilled water prior to staining with 0.1% (w/v) Coomassie Blue R-250 in 40%(v/v) methanol, 1% (v/v) acetic acid for 45 sec. The membrane was then destained with 50% (v/v) methanol and rinsed extensively with distilled water before excising the bands of interest and sent to sequencing.

3. 2. 11 Fixation and Staining Techniques

Protein bands could be detected without fixation by fluorescent labelling with low sensitivity. Mainly, gels were fixed for 60 min in 10% (w/v), trichloroacetic acid (Sigma), 5% (w/v) 5-sulphosalicylic acid (Sigma) in 40% (v/v) methanol. According to the amount of protein, gels loaded with about 3 to 1μg were stained with Coomassie and/or Periodic acid Schiff. Gels loaded with 0.1 μg to 1 μg were stained with silver or any other pre-staining treatment for silver.

3. 2. 11. 1 *o*-phthalaldehyde (OPA) and Coomassie blue Staining Methods

Fluorescent labelling with OPA was used for rapid detection of protein bands containing at least 5.0μg of protein. After running, gel was soaked in 50 ml sodium phosphate buffer 0.1 M pH 7.0 plus 2-mercaptoethanol (80 μl per 100 ml buffer). After 10 min, the buffer was changed with 50 ml of fresh buffer into which 20 mg *o*- phthaldialdehyde (Sigma) in 2 ml methanol was added. The gel was incubated in the dark for 15 min and examined under UV illumination at 365 nm.

When more sensitivity was required, the gel was fixed and then washed for 20 min (with two changes) in destaining solution [25% (v/v) ethanol, 8% (v/v) acetic acid]. Next, the gel was stained for 15 min in 0.5% (w/v) Coomassie blue R-250 (Sigma) dissolved in destaining solution. Finally, the gel was washed with several changes of destaining solution until background staining was removed.

For estimation of the molecular weight, the distance migrated by the molecular weight standards and distance migrated by the dye, were measured. Then a curve was generated by plotting the log of the molecular weight of standards *vs* relative mobility (Rf), defined as distance migrated by protein/distance migrated by dye. The Rf of proteins in the sample was measured and log of MW calculated with the equation of the curve.

3. 2. 11. 2 Silver Staining Techniques

The following methods were assessed: ammoniacal silver solution according to Oakley *et al.* (1980), silver nitrate by Merril *et al.*(1981), improved silver nitrate method of Blum *et al.* (1987) and improved silver nitrate by Rabilloud *et al.* (1988). Tables indicating the steps followed for completing these staining techniques are shown in appendices 10A,10B, 11A and 11B, respectively.

The best results were obtained with the improved method of Rabilloud *et al.* (1988). In short, after the fixation gels were rinsed three times for 30 min in 30% (v/v) ethanol, then they were equilibrated in MilliQ water for 15 min with one change. Next, gels were submerged for 1 min in sensitising solution (0.25 g/l sodium dithionite, Fisons Scientific) followed by two washes for 30 sec each, with MilliQ water. Gels were then impregnated with silver solution [0.2 % (w/v) silver nitrate (AnalR, DBH); 1 mM formaldehyde, (Sigma)] for 20 to 30 min.

After rinsing twice with MilliQ water for 1 min, gels were developed by immersing in 6% sodium carbonate (AnalR, BDH), 6 mM formaldehyde (Sigma), 20 μM sodium thiosulphate (AnalR, DBH). The reaction was stopped by adding acetic acid directly to the developer (3.5 ml per 100ml developer), while shaking for 10 min. Finally, gels were rinsed in water for 30 min and stored in 20% (v/v) ethanol at 4 °C and/or washed for 30 min in a solution of methanol 20% (v/v), 3% (v/v) glycerol and dried between cellophane sheets at 80°C for 1 to 2 h in a gel drier (model 1125B, BioRad). This silver staining procedure was effected after periodic acid oxidation (PA-silver), after periodic acid-Schiff staining (PAS-silver) and after staining with Alcian blue (AB-silver).

3. 2. 11. 3 Carbohydrate-specific Periodic acid Pre-staining Silver

In short, after fixation, gels were washed in fixation/destaining solution of 10% (v/v) acetic acid, 35% (v/v) methanol for 1 h. Next they were transferred to a freshly prepared

periodate solution (PA) [0.7%(w/v) periodic acid (Sigma) dissolved in 5% (v/v) acetic acid] where they were incubated for 1 h. After decanting, gels were briefly rinsed with water and submerged into freshly prepared sodium *meta*-bisulphite solution [0.2 g $Na_2S_2O_5$ (Sigma) in 100 ml 5% (v/v) acetic acid] for 5 to 10 min after which, solution was replaced until gel decolourised (another 5 to 10 min). At this point, silver staining started by rinsing the gels three times for 30 min in 30% (v/v) ethanol and then following the improved method of Rabilloud *et al.* (1988) in 3.2.11.2

3. 2. 11. 4 Carbohydrate-specific Periodic acid-Schiff (PAS) Staining Method

The protocol followed was as described by Carlsson (1993), which is based on the original method by Zacharius *et al.* (1969) for the staining of glycoproteins. After periodic acid oxidation and decolourisation described above, gels were placed into Schiff's reagent [0.45% (w/v) basic Fuchsin (Sigma) in 0.09 M HCl, 0.77% (w/v) sodium *meta*-bisulphite in water] and incubated until red bands appeared (0.5- 2h). The excess of reagent in the gel was removed by incubating in destaining solution described above. Gels were dried as described in 3.2.11.2. Preparation of Schiff's reagent is shown in appendix 12A

3. 2. 11. 5 Alcian Blue Pre-staining Silver for Glycoproteins

Alcian blue staining, originally developed by Wardi and Allen (1972), was used as a pre-staining step for silver. The protocol is described by Moller *et al.* (1993) and was improved by Moller and Poulsen (1995). The procedure is shown in appendix 12B.

After fixation, gels were washed in 5% (v/v) acetic acid for 4 min with 2 changes. Then, gels were place into freshly prepared periodate solution [1% (w/v) periodic acid (Sigma) dissolved in 5% (v/v) acetic acid] and incubated for 20 min. Next, washes in acetic acid were repeated as described above. This was followed by washes in water for 4 min with 2 changes and neutralization in 0.5 % (w/v) in potassium metabisulphite (Sigma) in water.

After these, the following steps were performed at 50 °C. First, two more washes of the gels with water for 4 min with 2 changes, followed by two washes for 4 min with 2 changes with destained solution [25% (v/v) ethanol, 10% (v/v) acetic acid in water]. Then, gels were stained for 15 min with 0.125% (w/v) Alcian Blue 8 6X (Sigma) dissolved in de-staining solution. Gels were destained with several changes of destaining solution at 50 °C until no bands or background staining could be seen. At this point, silver stain was started.

3.2.12 Glycoprotein Analysis

Preliminary characterisation of the glycan moiety of the purified enzymatic complex was made following the protocol of Manzi and Varki (1993) for quantification of free sialic acid (*N*-acetylneuraminic acid) and after enzymatic digestion and purification of bound sialic acid.

3. 2. 12. 1 Determination of Total Carbohydrate Content

Total carbohydrate content of samples was determined using the colorimetric method of Dubois *et al.* (1956). Samples of 40 µl were pipetted directly into the wells of a microtitre plate, and 40 µl of phenol [5% (w/v) in water, FSA Laboratory Supplies] was added followed by a fast delivered volume of 200 µl concentrated sulphuric acid (Fisons Laboratory Reagents). The reaction was left to stand for 10 min and then the whole plate was shaken for 10 sec and read on a Dynatech MR 5000-plate reader at 490 nm against appropriate blanks. A standard curve was produced using known concentrations of mannose (Sigma) in the range 0-80 µg (appendix 13A).

3. 2. 12. 2 Chemical and Enzymatic Deglycosylation

Chemical deglycosylation was attempted following the protocols given by Edge *et al.* (1981) and modifications according to Naim *et al.* (1988). A mixture of 1 ml anisole (Sigma) and 2 ml Trifluoromethanesulfonic acid (TFMS, Sigma) were prepared in a glass tube and cooled to 0°C on ice slurry. Six lyophilised samples (each containing 100 µg of the glycoprotein), were dissolved in 100 µl of the pre-cooled mixture in a 2-ml Ractivial (Pierce Chemical Co.). Nitrogen was bubbled through the solutions for 30 sec and put onto a CM-9 shaker (Sarstedt) for agitation on the ice slurry. Reactions were stopped at 5, 15, 30, 60, 90 and 120 min, by neutralizing with drop-wise addition of 1.2 ml of an ice-cold solution of pyridine-water (4:1). Finally, samples were dialysed over night *vs* Tris-HCl buffer 10 mM pH 6.0, concentrated on polyethylenglycol beds to 100µl and assayed for xylanase and arabinosidase activities.

Enzymatic deglycosylation was performed using the Prozyme deglycosylation kit, following manufacture's instructions but reducing all volumes 10 to 100 times. 10 µl 5X reaction buffer was added to 2.5 µl denaturing solution and mixed well to give 12.5 µl denaturing buffer. 1 µg of the samples (purified proteins, peaks from chromatograms) were lyophilised and resuspended in 4 µl of MilliQ water and put into PCR tubes. To each fraction, 1.25 µl denaturing-buffer was added and the mixture covered with two drops of mineral oil. Tubes were incubated a 100 °C for 5 min on a PTC 100 Programmable Thermal Controller (MJ Research Inc. GRI).

Tubes were left to cool down to room temperature while enzyme master mix was prepared, by mixing 10 µl of 5X reaction buffer, 18.5 µl sterile MilliQ water and 0.5 µl of each enzyme PNGase F, Sialidase A and Endo-O-Glycosidase. A diluted solution of Triton X-100 was made by adding 2.5 µl Triton solution to 22.4 µl sterile MilliQ water. To cooled samples, 2.5 µl of diluted triton solution were added and mixed thoroughly by pipetting down the oil layer. After this, 1 µl of enzyme master mix was added to each sample in the same way and the tubes were incubated at 37 °C fo 3 h. One of the tubes

was prepared with 1 µl diluted control glycoprotein (1µl bovine fetuin plus 99µl sterile MilliQ water, giving 100µl of 0.1 µg/µl protein).

After incubation, deglycosylation was assessed by the mobility shifts of the proteins on SDS PAGE revealed by Alcian Blue pre-staining silver, including deglycosylated and non-deglycosylated samples and controls. Mild-denatured procedure was also carried out by incubating the sample at 60°C for 5 min instead of the boiling step. In this case enzymatic digestion was extended up to 14 days at 37 °C.

3. 2. 13. 3 Determination of Enzymatic-released Sialic Acids

Determination of sialic acid (N-acetylneuraminic acid or NANA) was done by using the thiobarbituric acid assay as described by Manzi and Varki (1993). Preparation of reagents is shown in appendix 13B. It was necessary to produce de-*o*-acetylation of sialic acids prior to its enzymatic release in order to be detected by the colorimetric method. For this, 100 µg of purified fractions were lyophilised and dissolved in 40 µl of MilliQ water. The samples were then mixed with 40 µl of 0.2 M NaOH in the bottom of clean 2-ml glass tubes, which were then incubated at 37 °C for 0.5 h.

Once de-*o*-acetylation was completed, the mixtures were neutralized with 20 µl of 0.2 M H_2SO_4. Subsequent enzymatic release of sialic acid was effected by adjusting the buffer and following the Prozyme deglycosylation kit instructions but using only Sialidase A. After 3 h, samples were dialysed *vs.* a volume of 5 ml MilliQ water, overnight. For this, samples were pipetted into very small dialysis sacs floating on separate flasks. Then, the 5 ml dialysates and dialysed samples were lyophilised and dissolved in 60 µl of MilliQ water. Dialysed samples were used for protein determination (10 µl), for xylanase, arabinosidase and xylosidase activities (10 µl) and for total sugar content.

Dialysates (60 µl) were mixed with the periodate reagent [4.3% (w/v) sodium *meta*-periodate (Sigma), 58% (v/v) H_3PO_4(DBH) in water] and left to react for 20 min. Then,

250 µl of arsenite reagent [10% (w/v) soduim arsenite (Sigma), 7.1% (w/w) sodium sulphate (DBH) in 0.2 M H_2SO_4] was added with vigorous vortexing. Reactions were left to stand for 5 min. After these, 1 ml of TBA reagent [0.6% (w/v) 2-thiobabituric acid (Sigma), 7.1% (w/v) sodium sulphate (DBH) in water] was added to each reaction tube, mixed well and heated up to 100 °C for 15 min. Finally, 1 ml of cyclohexanone (Aldrich) was added and vortexed well to extract the chromophore. The mixture was chilled on ice for 2-3 min and centrifuged at 1,000 x g at room temperature for 5 min. The absorbance of the organic solvent supernatants were read at 549 nm (sialic acid chromophore absorbance max) and at 532 nm (interfering chromophore absorbance max) against blank samples treated in identical fashion. A standard curve was produced using *N*-acetylneuraminic acid (Sigma) in the range of 1-10 nmol (appendix 13C).

3. 3 Results

In order to gain possible evidence for the involvement of arabinosidase and xylanase in the pathogenesis and/or virulence of *Stagonospora nodorum* towards wheat, and to understand how these enzymes might operate against host cell walls, experiments were set up with the aim of creating a protocol for the purification and further characterisation of these CWDE from liquid fluids. Separation would have enable studies to be conducted on their abilities to degrade host cell walls, both alone and in combination. Possible synergy may exist between the two activities but also between arabinosidases and the major wall protein-degrading trypsin of *S. nodorum* (Carlile *et al.*, 2000) in view of the protective arabinoside side chains that abound on cell wall extensins.

Throughout many different separation techniques, fractions retained arabinosidase and xylanase together. Further experiments were carried out in order to understand the nature of the association between these two activities and to evaluate if the activities could be separated.

3. 3. 1 Protein Fractionation and Ion Exchange Chromatography

Precipitation serves to achieve concentration but, in mixtures, like extracellular enzymes from young liquid cultures, a degree of separation and purification can be achieved by the conversion of solutes to solids according to differences in solubility. Ammonium sulphate fractionation of liquid culture supernatants from *S. nodorum*, growing on wheat cell wall as a single carbon source, gave xylanase and arabinosidase activities at the levels presented in Table 3. Proteins remaining in solution in the 65% fraction showed the highest total and specific activities for xylanase and arabinosidase, followed by the 35% fraction. In terms of purification with the fractions of 65% and 35%, xylanase could be

purified 4.79 and 3.52 respectively and arabinosidase could be purified 1.9 and 1.28 respectively, in a single step.

Table 3. Ammonium sulphate fractionation of culture filtrates for (a) xylanase activity and, (b) arabinosidase activity. Each fraction represents percentage of ammonium sulphate saturation at 0° C.

(a) Xylanse activity

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity per µl (n Kats)	Specific activity per mg (nKats)	Purification (n-Fold)
80%	50.0	1.67	83.5	0.95	0.57	1.68
65%	50.0	3.30	165.0	5.40	1.63	4.79
55%	50.0	1.63	81.5	1.36	0.83	2.44
45%	50.0	1.48	74.0	1.12	0.76	2.23
35%	50.0	2.37	118.5	2.85	1.20	3.52
15%	50.0	2.60	130.0	2.53	0.98	2.88
Culture filtrate	500.0	1.58	790	0.54	0.34	1.00

(b) Arabinosidase activity

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (n Kats)	Specific activity per mg (nKats)	Purification (n-Fold)
80%	50.00	1.67	83.5	0.03	0.01	0.02
65%	50.00	3.30	165.0	2.44	0.74	1.90
55%	50.00	1.63	81.5	0.63	0.39	1.00
45%	50.00	1.48	74.0	0.58	0.39	1.00
35%	50.00	2.37	118.5	1.20	0.50	1.28
15%	50.00	2.60	130.0	0.36	0.14	0.36
Culture filtrate	500.00	1.58	790	0.62	0.39	1.00

For the next step aiming for eventual purification of xylanase isoforms, ion exchange chromatography was chosen. This was because the information given by preparative IEF with the Rotofor system indicated that xylanase isoforms were present in the culture filtrate in the wide range from pH 5 to pH 9. In order to fractionate the isoforms, pH of the protein solution was reduced stepwise, obtaining the differentially charged isoforms at every given pH of the solution. Using this procedure it was expected that the molecules could be separated according to their charge on exchange suports. Therefore, it was

expected that around pH 8 to 9 the basic xylanase isoform would be positive and then would bind to the cation exchanger.

Initially, chromatography was performed with a protein sample (65% ammonium sulphate fraction) of 6 mg in the cation exchanger SP Sepharose FF. Running was done with 25 mM borate pH 9.0 buffer, with a gradient made of 60 ml buffer and 60 ml buffer plus 0.3 M NaCl. The result was that protein exhibiting arabinosidase and xylanase activities did not bind to the support and few, non-active proteins, bound the column.

A second chromatography was performed with the same sample but using the cation exchanger AG MP 50. For running the pH was reduced with 25 mM Tricine buffer pH 8.0. Under these conditions, most of the protein fraction (with xylanase and arabinosidase activities) did not adsorb to the support and appeared in the elution buffer (Fig 20). A small proportion of the protein fraction (containing xylanase activity) adsorbed to the support and eluted with the sodium chloride gradient. Both, bound and unbound fractions were run on preparative isoelectricfocusing and assayed for xylanase activity (Figures 20b and 20c). Bound fraction gave one xylanase isoform with pI 8.3. It was assumed that this peak was the basic xylanase partially purified previously by this group, and therefore no further action was taken.

The unbound fractions gave what seemed to be seven protein peaks all with xylanase activity with pIs 3.0, 4.0, 4.6, 4.8, 5.1, 5.5, and 6.0. All of them showed xylanase activity. Arabinosidase activity was not measured but according to previous information (Chapter 2), it was expected that two out of the seven peaks would have arabinosidase activity as well. At this point, the approach of fractionating and separating the proteins by reducing the pH of the solution and performing ion exchange appeared successful.

A third chromatography was performed with the same sample running in the cation exchanger SP Sepharose FF and using 50 mM succinate buffer pH 5.8. The result was that most of protein fraction (with xylanase and arabinosidase activities) was washed out from the column, while the smaller bound protein fraction did not show any activity. This

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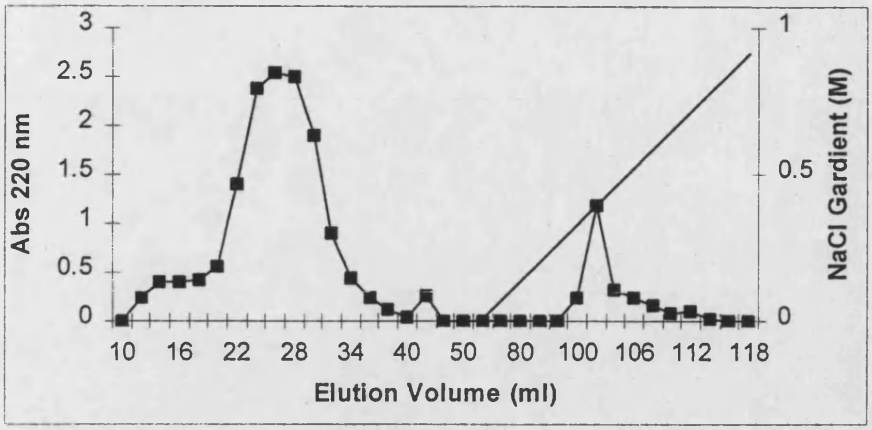
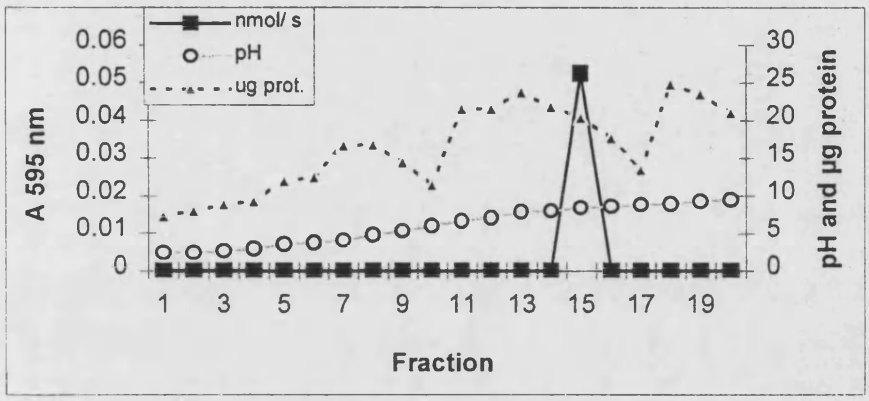
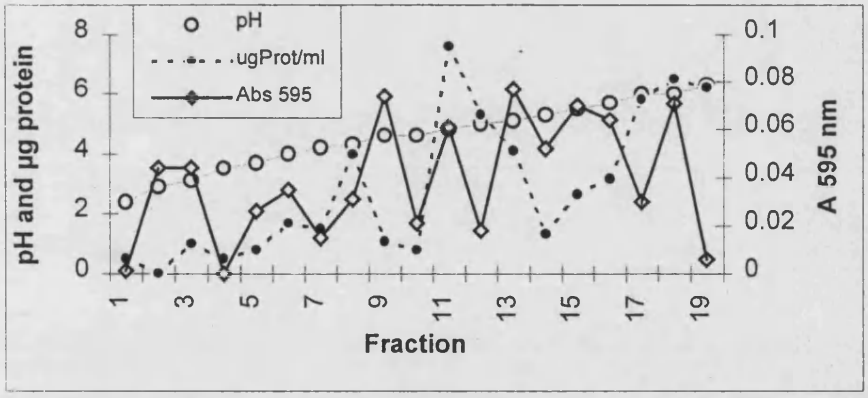


(a) Cation exchange chromatogram at pH 8.0. Protein detected at A 220 nm



(b) Isolelectric focusing of bound protein. Enzymatic activity as A at 595 nm



(c) Isolectric focusing of unbound proteins. Enzymatic activity as A at 595 nm

Figure 20 : Xylanase isoforms of bound and unbound fractions to the cation exchanger AG MP 50 at pH 8.0. (a) chromatogram of the 65% Ammonium sulphate fraction run of the cation exchanger; (b) the rotoford isoelectric focusing patterns of bound; and (c) unbound proteins assay with RBB-Xylan substrate. Bound fraction gave one xylanase isoform pI 8.3.

was unexpected since it was expected that, at least the same basic xylanase bound in the previous chromatography run (pI 8.3 in preparative IEF) should have bound to the support.

Next, the pH of the sample was reduced, and run with 50 mM succinate buffer pH 5.6 and pH 5.4 respectively. Again and unexpectedly, neither xylanase nor arabinosidase bound to the column. A similar result was obtained when chromatography was run in reduced pH and ion strength, working with 25 mM succinate buffer pH 5.0. This suggested that the ionic strength of the buffer was not the cause for the inability of the proteins to bind to the SP Sepharose FF.

In subsequent experiments, the support was changed in order to assess the effect of this variable. The cation exchanger AG MP 50 (which was successfully used in the second chromatography) was run with 20 mM succinate buffer pH 5.4 and with acetate 20 mM buffer pH 4.6. Under these conditions, it was expected that the basic xylanase with pI 8.3 and the acidic xylanases (pI's 6.0, 5.5, and 5.1) would bind to the support. However, as observed in the previous experiments, xylanase and arabinosidase did not absorb to the support.

It was then decided to use anion exchangers working on a pH around 5.0. It was expected that xylanases with pI's 8.3, 6.0 and 5.5 would be eluted in the washing buffer and the other xylanases would bind to the column. A sample, containing 8.0 mg protein was loaded on a Q Sepharose Fast Flow column and ran with 20 mM succinate pH 5.0 buffer. The gradient was composed of 60 ml elution buffer and 60 ml elution buffer plus 0.3 M NaCl. The result was again unexpected since all xylanase and arabinosidase activities were present in the major bound protein fraction, while the smaller protein fraction that was washed out, did not show any activity.

The anion exchange support was next changed and the pH of the buffer reduced. Samples containing 8 mg of protein were loaded in the resin AG MP-50 and ran with 20 mM acetate buffer pH 4.6 and pH 4.0. In each case the result was similar. The unbound

protein fractions did not show any enzymatic activity and the NaCl-eluted fraction contained both xylanase and arabinosidase activities.

At this point, it was decided that ion chromatography was not an appropriate technique for the separation of this CWDEs. The question remained as to why there was no change in the charge of the putative isoforms as separated by the preparative IEF and why they were adsorbed or eluted together.

3. 3. 2 Affinity Chromatography

The next attempt in the purification of xylanases and arabinosidases involved the use of affinity supports. With this aim in mind, xylan and concanavalin A were assessed as affinity ligands for xylanases and/or arabinofuranosidases. It was found that protein fractions exhibiting both arabinosidase and xylanase activities bound to birchwood xylan while they did not bind to concanavalin A. This result indicated that either both proteins were not glycosylated or that the concanavalin A lectin did not recognise and bind the putative sugar moiety of the proteins.

In view of the capacity of substrates to bind the enzymes, crosslinked substrates were prepared and conditions for binding were standardised. The results indicated that both activities remained together, adsorbing in a similar pattern to all the supports (Fig 21 and Fig 22 for xylanase and arabinosidase respectively).

The adsorption of both enzymes to the four supports was influenced by pH and ionic strength of the buffers. The pattern of adsorption showed a minimum binding at around pH 5.0 and two maxima at the lowest and the highest pH's studied. Adsorption of xylanase activity was maximal in cross-linked birchwood xylan at pH 3.7. Adsorption of arabinosidase activity to commercial arabinan (Megazyme) was maximal at pH 8.0.

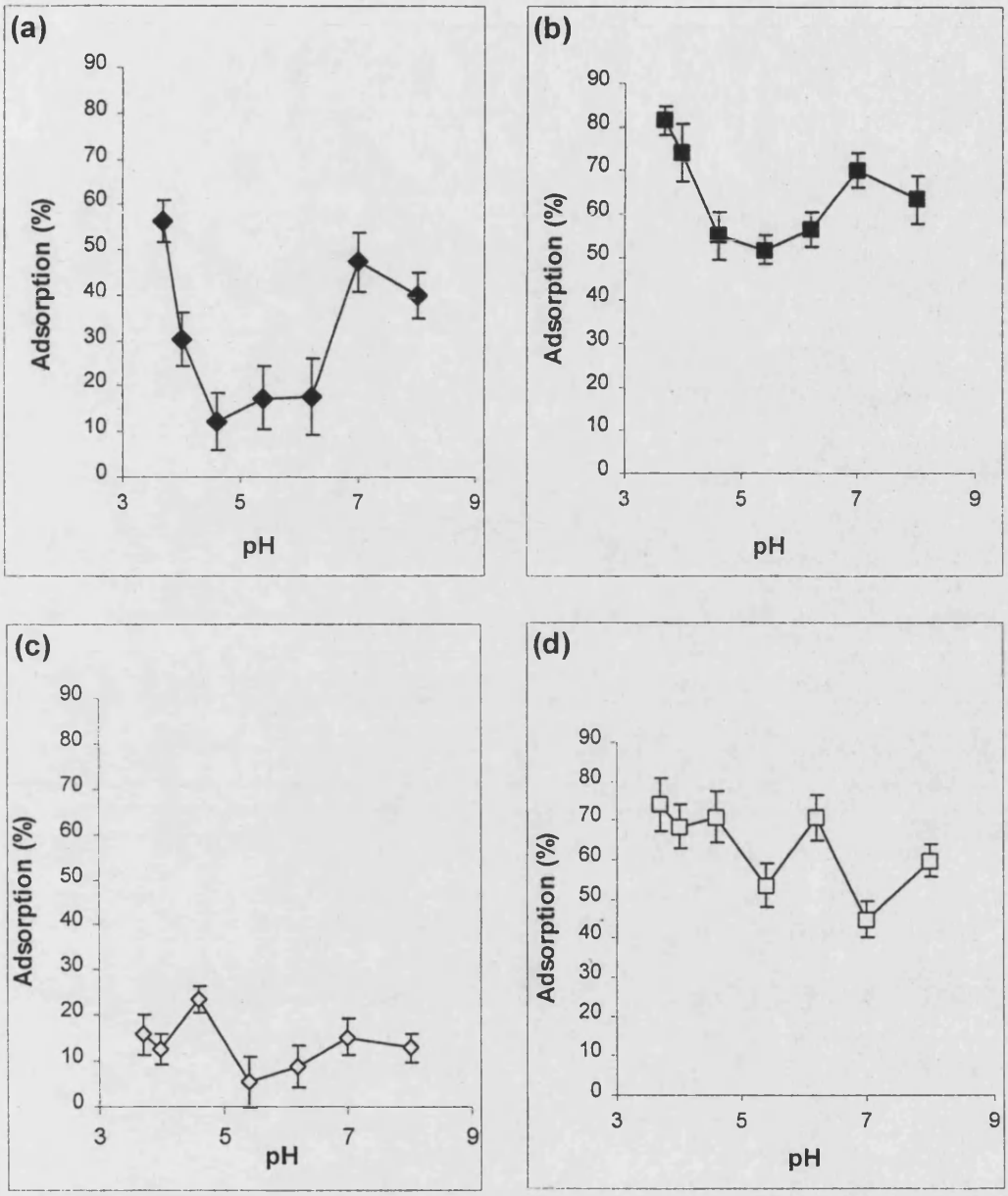


Figure 21 : Adsortion of xylanase to cross-linked substrates: (a) oat spelt xylan (OX); (b) birchwood xylan (BX); (c) Lab-araban (LA); and (d) Megazyme arabinan (MA).

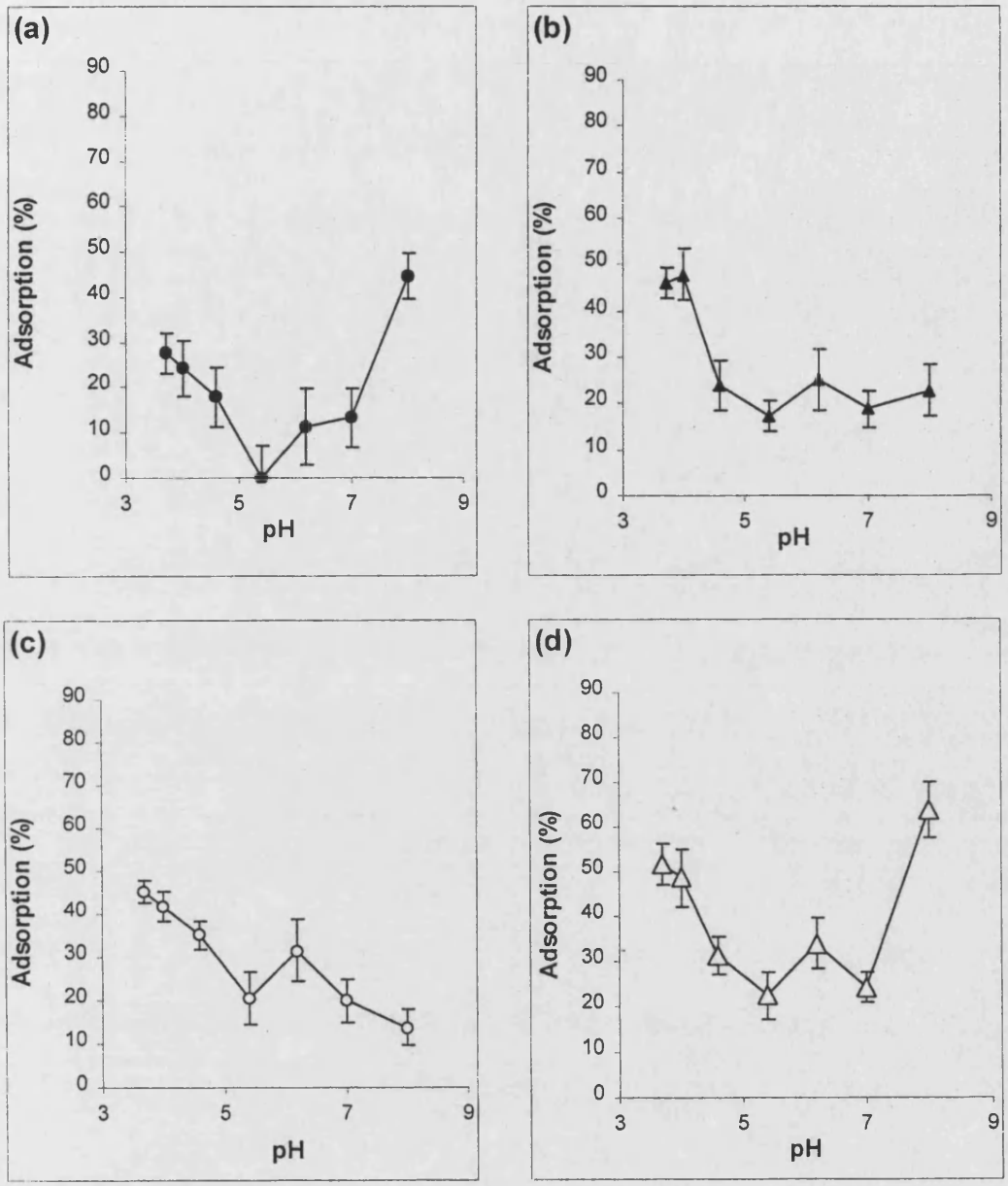
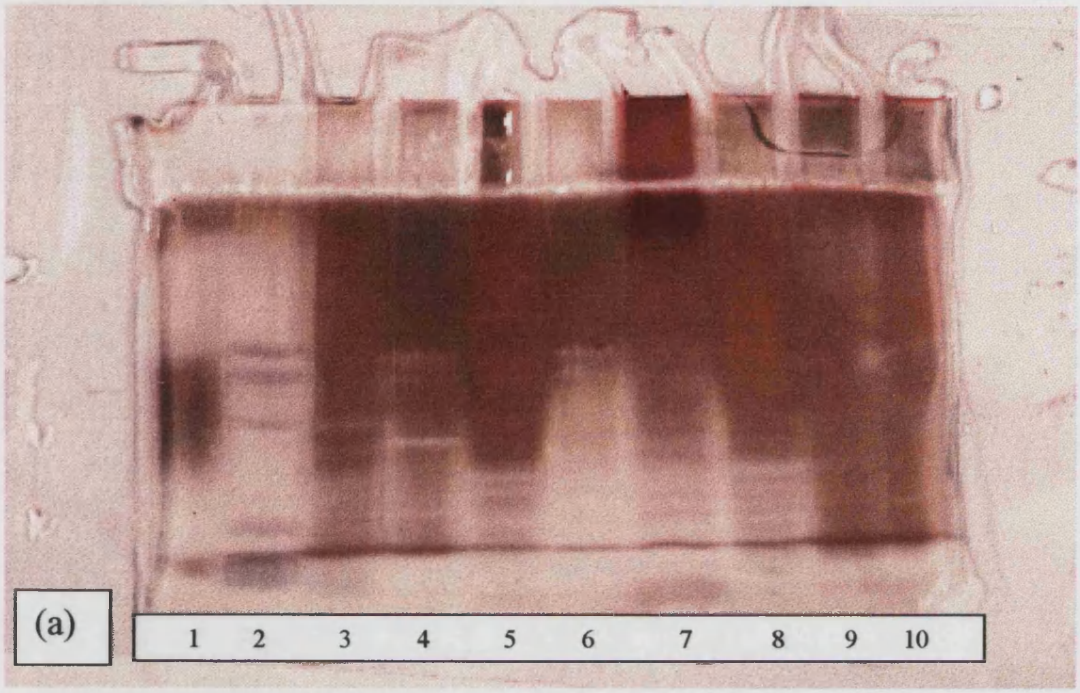


Figure 22 : Adsortion of arabinosidase to cross-linked substrates: (a) oat spelt xylan (OX); (b) birchwood xylan (BX); (c) Lab-araban (LA); and (d) Megazyme arabinan (MA).

Bound fractions to cross-linked xylans and cross-liked arabinan were electrophoresed, pre-stained with periodic acid-Schiff (PAS, see 3.2.11.3) and finally stained with silver (Fig 23a). With this procedure, the presence of polysaccharides in the samples was revealed. Part of these saccharides could be removed with the use of desalting columns in harsh denaturing conditions (see 3.2.8.1). After electrophoresis of the "clean" fractions and periodate oxidation pre-staining silver for glycoproteins (PA-silver, see 3.2.11.2) it was revealed that all the supports bound less proteins at the extreme pHs. Fig 23b shows more bands in the SDS-PAGE of the fractions bound at pH 5.0 to 7.0 than in the fractions bound at pH 3.7, 4.0 or 8.0. Among the supports, the araban prepared here (Lab-araban) and run at pH 8.0 showed the least number of bands.

Bound fractions to lab-araban at pH 8.0, Megazyme arabinan at pH 3.7, birchwood xylan at pH 3.7, pH 4.0 and pH 8.0 were chromatographed through gel filtration under mild denaturation (see 3.2.8.2) and then electrophoresed under denaturing conditions (SDS-PAGE). The gels were then stained only with silver or pre-stained with PA followed by the dye Alcian Blue before silver staining (see 3.2.11.4). This technique revealed that protein bands contained tightly bound polysaccharides not observed when silver without pre-staining was used (Fig 24a and Fig 24b). Also, the Alcian blue revealed more clearly the protein bands than the gel stained only with silver. This means that the proteins were glycosylated and that they contained acidic polysaccharides, which impaired the usual silver staining and were revealed by the cationic dye.

The pHs at which there were fewer bands and more xylanase or arabinosidase activities were 8.0, 4.0 and 3.7. Chromatography was therefore performed at these pHs. Typical chromatograms using the crosslinked substrates Megazyme-arabinan, lab-araban and birchwood xylan are presented in Fig 25.



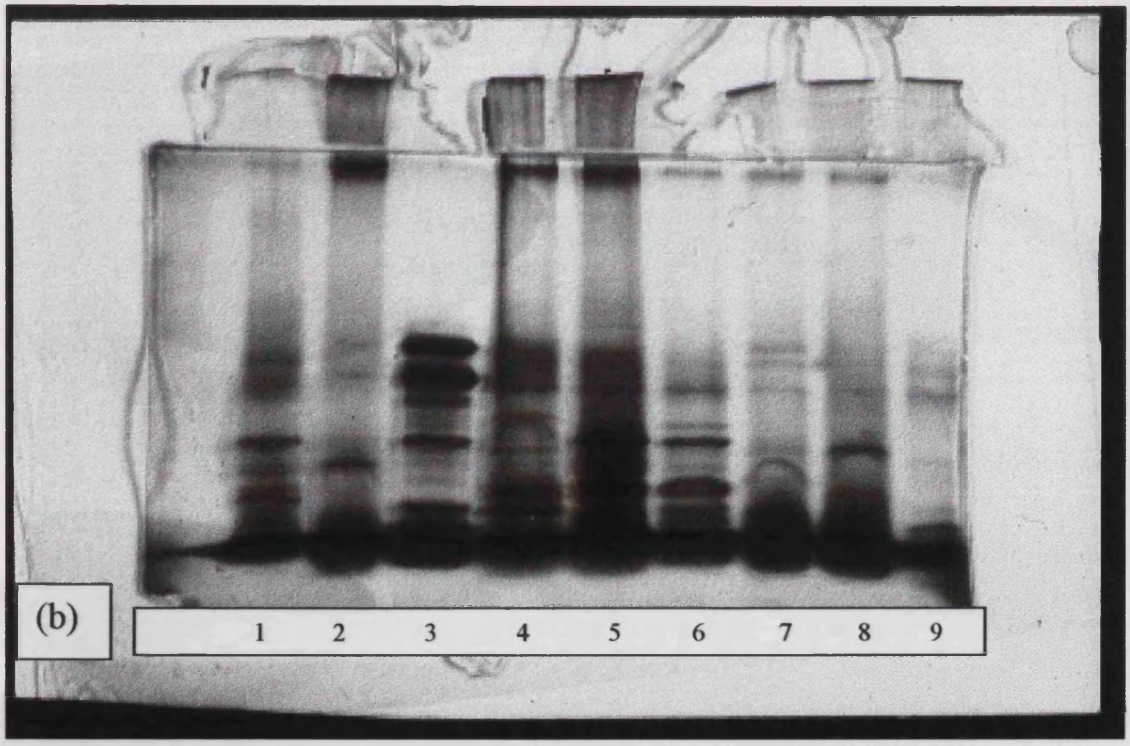


Figure 23 : SDS PAGE of protein fractions bounded to cross-linked substrates. (a) Gel pre-stained with PA-Schiff and then with silver. 1, glycosylated marker; 2, non-glycosylated marker; 3-10, protein fractions bounded to crossed-linked substrates showing high content of sugars; (b) Same fractions after gel filtration under harsh denaturing conditions and PA pre-staining silver. 1, LA pH3.7; 2, BX pH4.0; 3, marker (SBA); 4, OX pH4.0; 5, MA pH5.0; 6, OX pH6.0; 7, BX pH7.0; 8, LA pH8.0; 9, MA pH8.0. LA = Lab-araban, MA = Megazyme arabinan, BX= Birchwood xylan and OX = Oat spelt xylan.

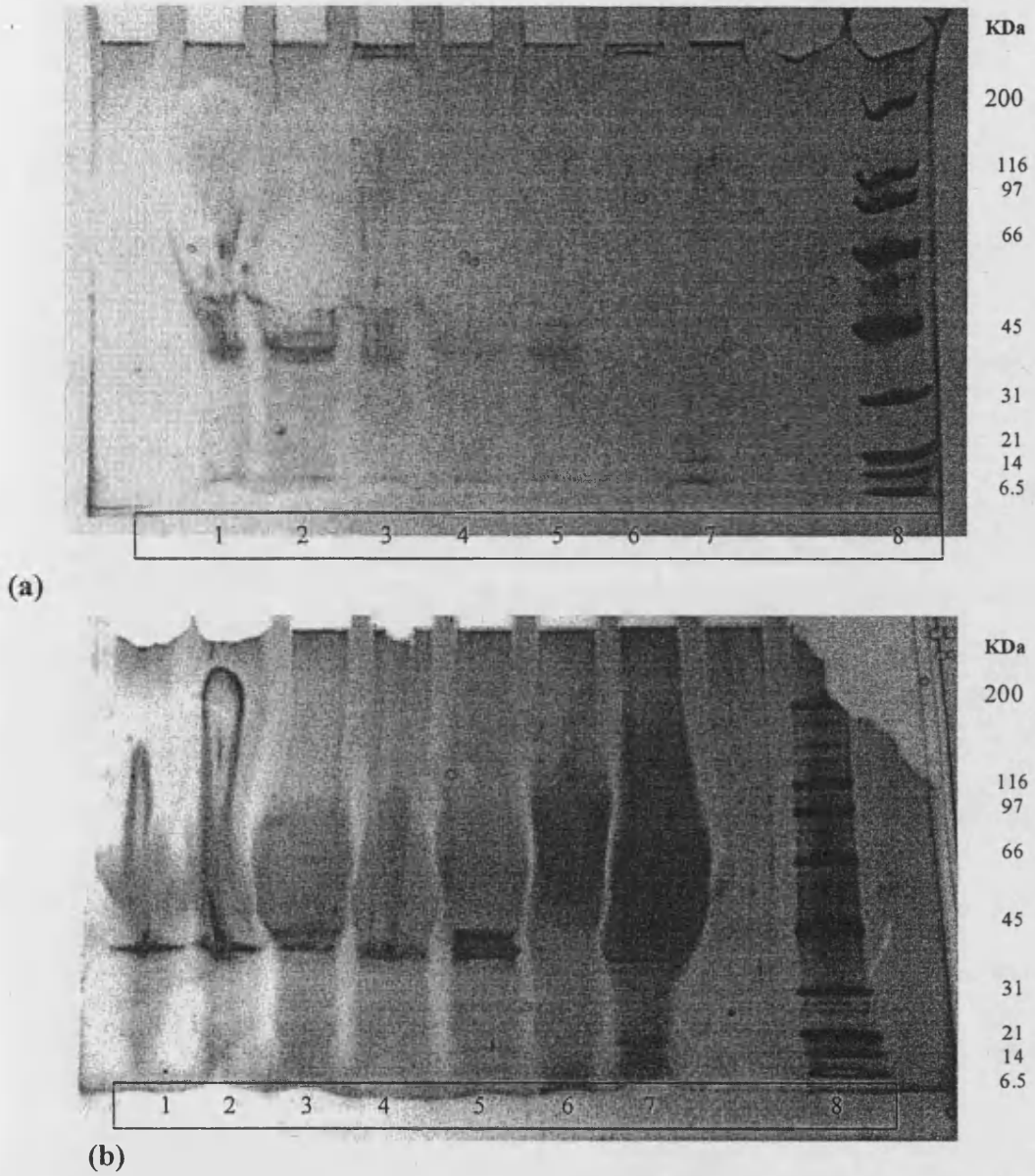
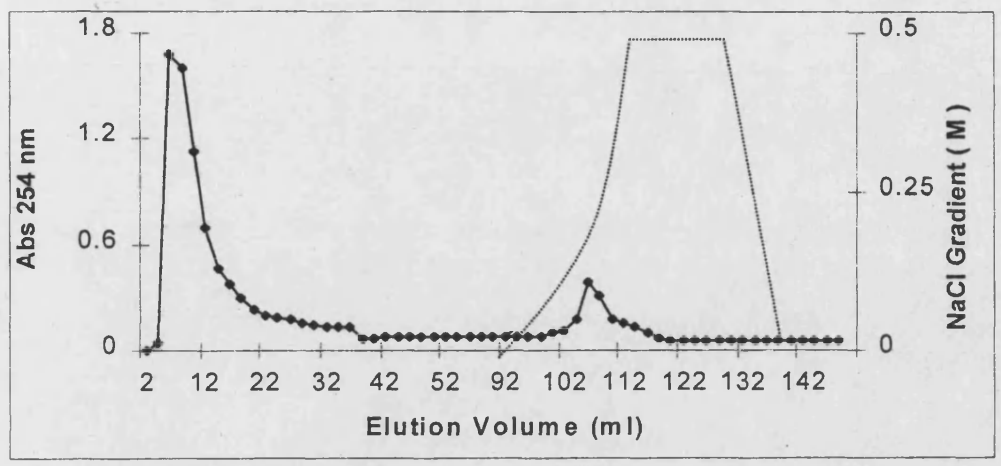
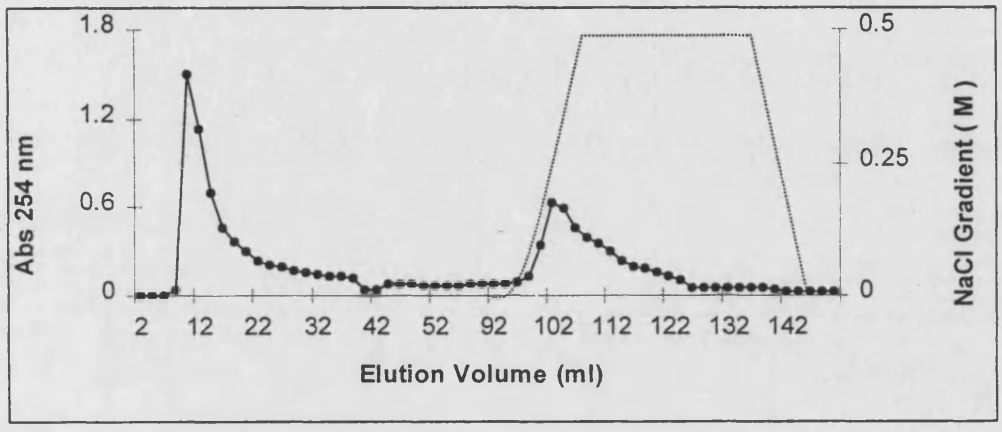


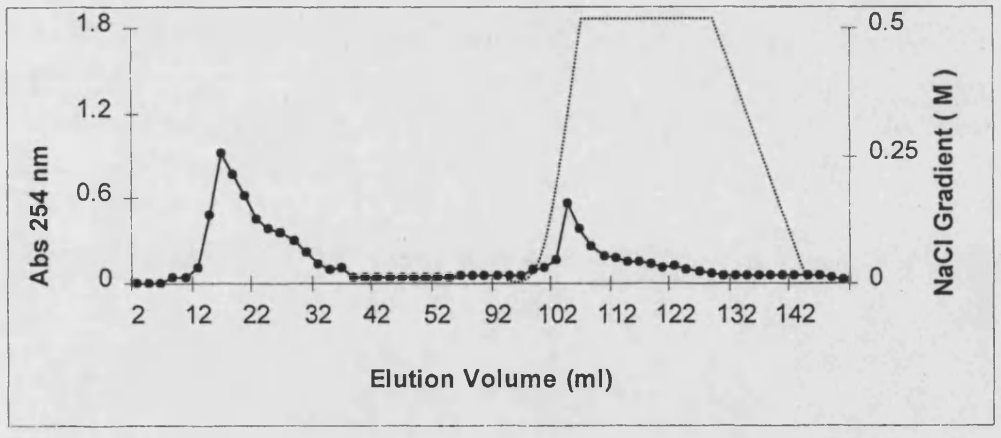
Figure 24 : SDS PAGE of protein fractions bounded to cross-linked substrates, after gel filtration under mild denaturing conditions. (a) Gel stained only with silver and (b) Same protein fractions pre-stained with PA-Alcian Blue and then with silver: 1 and 2, LA pH8.0; 3 and 4, MA pH3.7; 5, BX pH3.7; 6,BX pH 4.0; 7, BX pH 8.0 and 8 molecular mass standard. LA = Lab-araban, MA = Megazyme arabinan and BX = Birchwood xylan



(a)



(b)



(c)

Figure 25 : Affinity chromatography on crosslinked substrates at different pHs. (a) chromatogram of croslinked lab-arabinan at pH 8.0; (b) chromatogram of crosslinked birchwoodxylan at pH 4.0 and (c) chromatogram of crosslinked Megazyme arabinan at pH 3.7. Protein was detected at 254 nm.

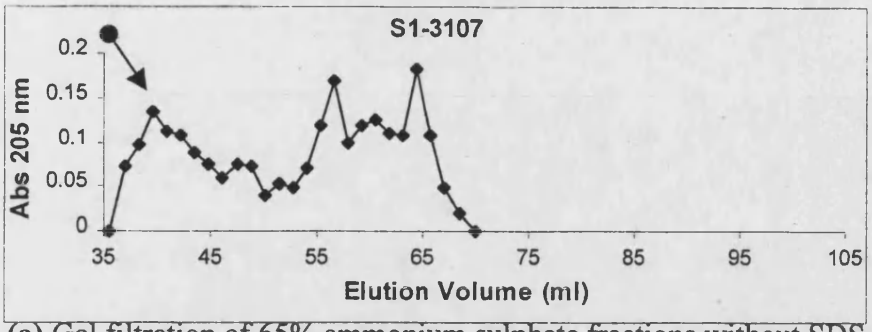
3. 3. 2 Gel Filtration Chromatography

Because affinity chromatography resolved few protein bands and they appeared to be different enough to be separated by size, and also because of the presence of polysaccharides in the samples, gel filtration chromatography was performed as a purification step for crude extracts and for affinity fractions.

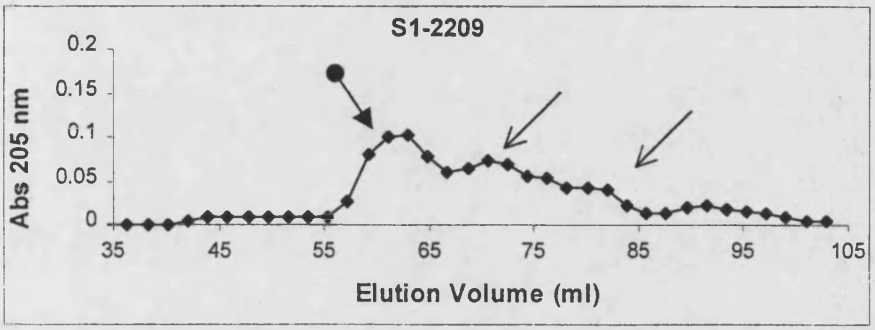
Culture filtrate protein samples concentrated by ammonium sulphate precipitation (65% saturation) and affinity chromatography-purified fractions were both used in gel filtration chromatography on Sephacryl S-100.

When the ammonium sulphate fractions were used on samples without further treatment (Fig 26a for column 1 and Fig 27a for column 2), few peaks were resolved. The first peak that appeared after the dead volume contained both xylanase and arabinosidase activities. The elution volume for this peak was different in each column and was not consistent in consecutive runs. It had a molecular weight ranging from 293 to 172 kDa calculated with the use of the standardisation curves constructed for both columns (see 3.2.8.3 and appendix 14).

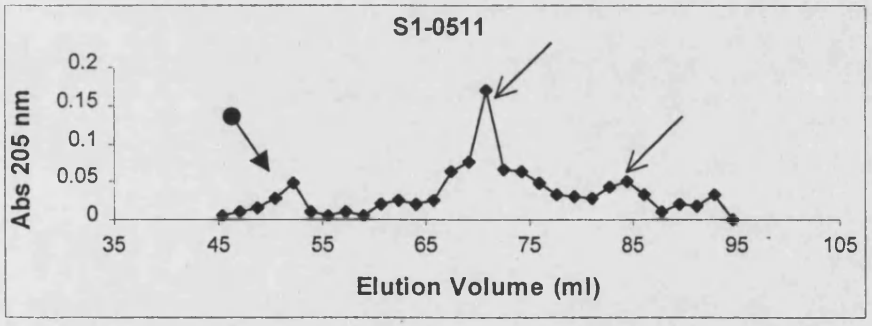
Attempted separation of arabinosidase from xylanase activity from 65% ammonium sulfate fractions involved treatment of the samples with SDS and β-mercaptoethanol at 60 °C for 10 min and running in the presence of the detergent and the reducing agent in the elution buffer. Under such conditions, the first peak and in some cases the second peak, contained both xylanase and arabinosidase activities. These peaks were followed by others showing just xylanase activity (see Fig 26 and Fig 27). Nevertheless, the number of each of these peaks and their elution volume were not consistent or repeatable in consecutive experiments in any of the two columns, comprising elution volumes from 50 to 85 ml (Fig 26b and Fig 26c for column 1 and Fig 27b and Fig 27c for column 2).



(a) Gel filtration of 65% ammonium sulphate fractions without SDS

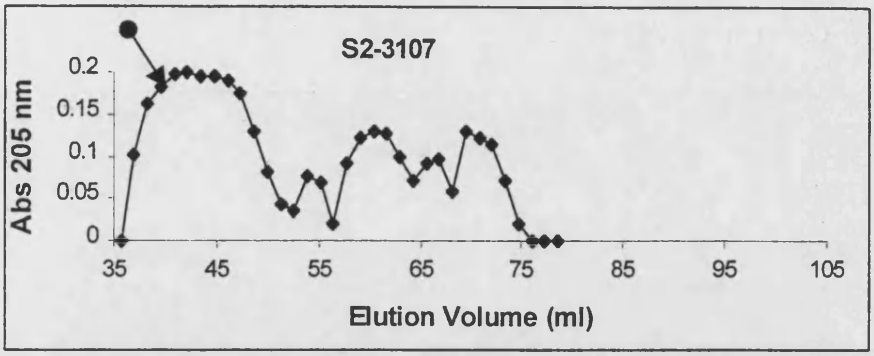


(b) Gel filtration of 65% ammonium sulphate fractions in mild denaturation

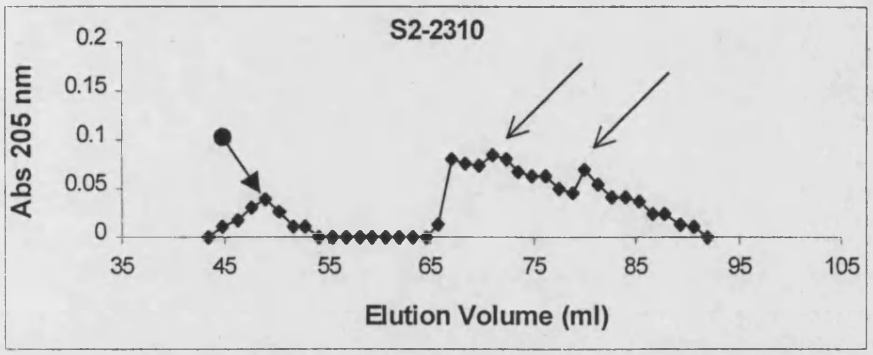


(c) Gel filtration of 65% ammonium sulphate fractions in mild denaturation

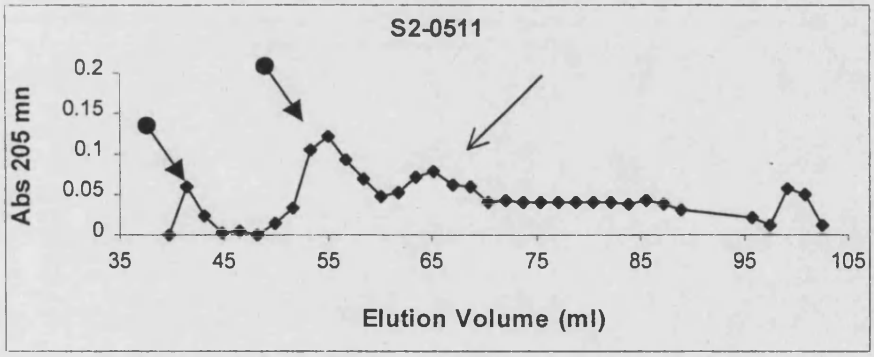
Figure 26 : Gel filtration chromatograms of 65% ammonium sulphate fraction on Sephacryl S-100, column 1. 500 µg of protein were dissolved into 60 µl loading buffer, heated to 60 °C for 5 to 10 min, laid onto the gel and run at 0.25 ml/min flow. (a) Loading and elution buffer were 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl; (b) and (c) Loading buffer was 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl, 1% (w/v) SDS, 280mM 2-mercaptoethanol. Elution buffer was 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl, 0.1% (w/v) SDS, 1mM 2-mercaptoethanol. Arrows indicate peaks with both arabinosidase and xylanase activities (●⟶) and just xylanase activity (⟵). Protein was detected at 205 nm.



(a) Gel filtration of 65% ammonium sulphate fractions without SDS



(b) Gel filtration of 65% ammonium sulphate fractions in mild denaturation



(c) Gel filtration of 65% ammonium sulphate fractions in mild denaturation

Figure 27 : Gel filtration chromatograms of 65% ammonium sulphate fraction on Sephacryl S-100, column 2. 500 µg of protein were dissolved into 60 µl loading buffer, heated to 60 °C for 5 to 10 min, laid onto the gel and run at 0.25 ml/min flow. (a) Loading and elution buffer were 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl; (b) and (c) Loading buffer was 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl, 1% (w/v) SDS, 280mM 2-mercaptoethanol. Elution buffer was 20 mM Tris-Cl pH 6.0 plus 0.2M NaCl, 0.1% (w/v) SDS, 1mM 2-mercaptoethanol. Arrows indicate peaks with both arabinosidase and xylanase activities (● ↘) and just xylanase activity (↙). Protein was detected at 205 nm.

The sizes of the peaks with arabinosidase and xylanase activities ranged from 151 to 75 kDa (151, 129, 108, 75). The peaks with just xylanase activity had molecular weights ranging from 55 to 26 kDa (e.g. 55, 53, 51, 48, 35, 29 and 26 kDa).

When the affinity-purified fraction (crosslinked lab-arabinan at pH 8.0) was used for gel filtration, after treatment with SDS and reducing agent, the bound xylanase-arabinosidase complex was usually resolved in two peaks. Both peaks, showed again arabinosidase and xylanase activities, though they eluted at different volumes and had molecular weights ranging from 130 to 27 kDa. They showed different degrees of separation independent of the quantity of protein loaded into the column. However there was a tendency of increased molecular weight with increased protein loaded, as shown in Fig 28.

It was evident that arabinosidase was closely associated with xylanase since separation of both activities by different means were unsuccessful. Gel chromatography using the affinity fractions was carried out several times. The peaks were pooled together in different groups according to their elution volume and aliquots from each group were assayed for arabinosidase and xylanase activities. Activities of several runs were compared and presented as scale in Table 4. It was noticed that the arabinosidase activity diminished as the xylanase activity increased in each group and this occurred concomitantly with increasing elution volume (or decreasing molecular size) of the proteins containing the enzymatic activities.

Table 4. Sizes, activities and sugar contents of fractions from gel filtration chromatography.

Group	Elution Volume (ml)	Molecular Weigh (KDa)	Arabinosidase Activity	Xylanase Activity	Carbohydrate Content % (w/w)
1	42 - 48	140 - 110	+++	++	60
2	50 - 55	100 - 80	++	++	56
3	62 - 65	62 - 55	+	++	54
4	69 - 73	47 - 39	_	+++	47
5	76 - 80	35 - 29	_	+++	42
6	82 - 86	27 - 23	_	++	37

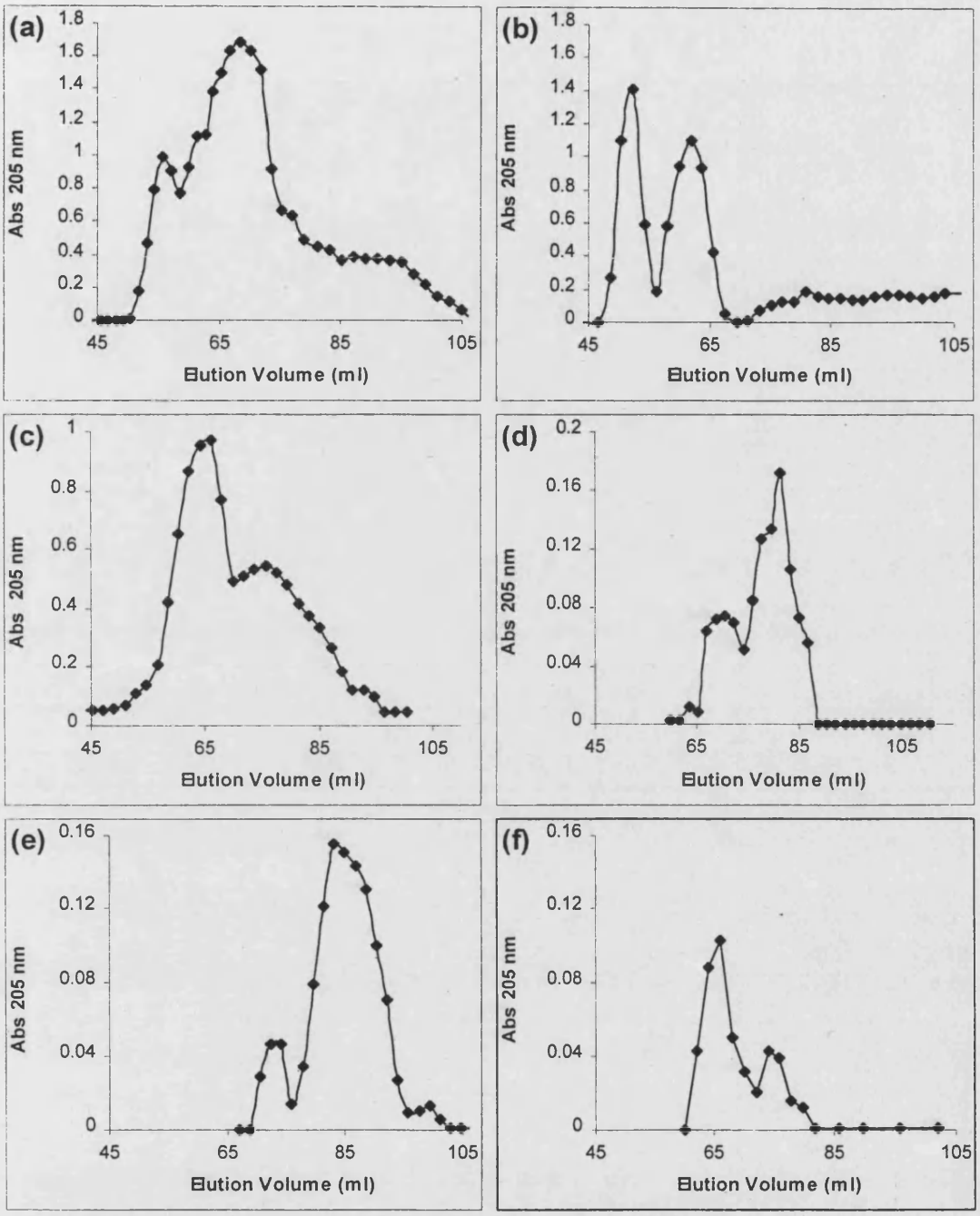


Figure 28 : Gel filtration chromatograms of crossliked lab-araban affinity fractions on Sephacryl S-100, column 2. Protein was dissolved into 60 µl loading buffer, heated to 60 °C for 5 to 10 min, laid onto the gel and run at 0.25 ml/min flow,Loading buffer was 20 mM Tris-Cl pH 6.0, 0.2M NaCl, 1% (w/v) SDS, 280mM 2-mercaptoethanol. Elution buffer was 20 mM Tris-Cl pH 6.0, 0.2M NaCl, 0.1% (w/v) SDS, 1mM 2-mercaptoethanol. Protein loaded was: (a) 500 µg; (b) 450 µg; (c) 400 µg; (d) 350 µg; (e) 300 µg; and (f) 250 µg. Both peaks showed both arabinosidase and xylanase activities. Protein was detected at 205 nm.

3. 3. 4 Hydroxylapatite Chromatography

In a new attempt to separate both activities, hydroxylapatite (HA) was used as a chromatography support to separate xylanase from arabinosidase fom the affinity purified fraction. The mechanisms of protein-HA interaction are more complex than for other types of chromatography, nevertheless it has been used successfully to separate glycoproteins containing sialic acid residues (Gorbunoff, 1980).

With the buffer system employed, it was expected that the protein would emerge from the column according to their charge in the following order; first the more acidic, followed by the neutral proteins and then the mixture of acidic-basic molecules (starting from the more to the less acidic). Finally, the predominantly basic proteins bound to the column would elute with the highest sodium chloride concentration.

The results obtained showed that xylanase and arabinosidase activities were both present in the first eluted fraction (more acidic molecules, Fig 29) and in the loosely adsorbed fraction (a mixture of differently charged molecules). The tightly bound fraction did not show enzymatic activity. This was in favour of a glycosylated complex holding charge and expressing polydispersity in respect to that charge.

3. 3. 5 An improved Method for the purification of the Enzymatic Xylanolytic Complex of *Stagonospora nodorum*.

The procedures listed in Table 5 summarize the steps that were used for the purification of the multienzymatic xylanolytic complex of *S. nodorum*. The complex contained endoxylanase, α-arabinofuranosidase and β-xylosidase activities, measured by the Remazol Brillian Blue-Xylan assay, *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-xyloside derivatives, respectively.

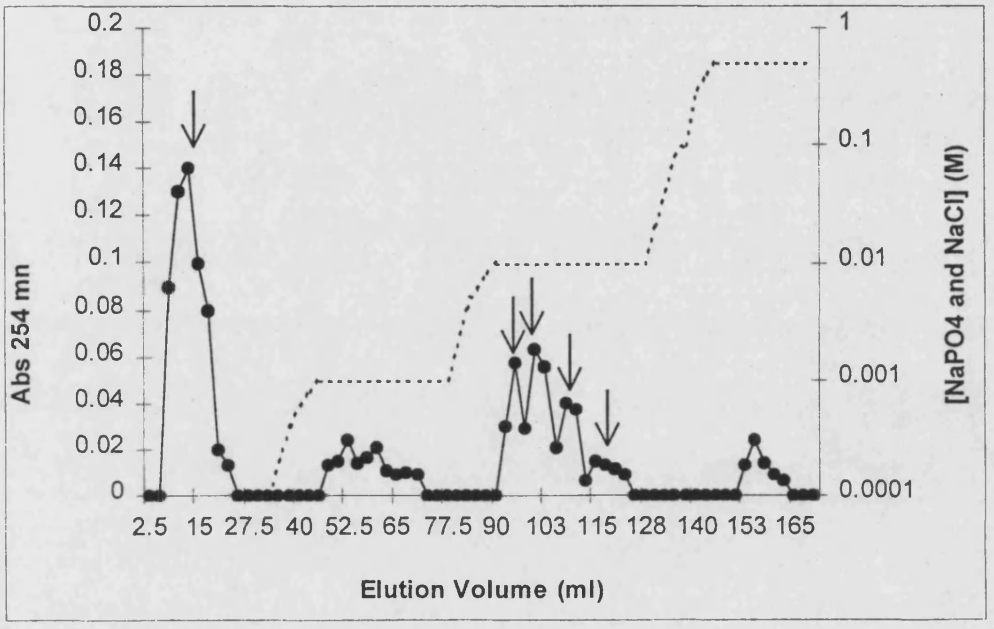


Figure 29: Hydroxylapatite chromatography of the affinity purified fraction. Arrows indicate the fraction with arabinosidase and xylanase activities.

Table 5. Steps in purification of the enzymatic complex arabinosidase-xylanase

(a) Xylanase Activity

Purif. Step	Protein µg / µl	Sugar Content % (w/w)	Sialic Acid % (w/w)	Volume (ml)	Total Protein (mg)	Activity per µl (nkats)	Total Activity (µkats)	Specific Activity	Purif Fold
Culture Filtrate	0.9	72	ND	1000	900	0.43	387	0.43	1.0
65% Amm. Sulphate Fraction	3.3	82	ND	50	165	5.3	265	1.6	4.0
Affinity chromato.	0.2	76	16	2.0	0.4	17	34	85	193
Gel Filtration (*)	0.16	47	8.4	2.0	0.32	15	30	93	211

(b) Arabinosidase Activity

Purif. Step	Protein µg / µl	Sugar Content % (w/w)	Sialic Acid % (w/w)	Volume (ml)	Total Protein (mg)	Activity per µl (nkats)	Total Activity (µkats)	Specific Activity	Purif. Fold
Culture Filtrate	0.9	72	ND	1000	900	0.45	405	0.45	1.0
65% Amm. Sulphate Fraction	3.3	82	ND	50	165	2.5	125	0.75	1.7
Affinity chromato.	0.2	76	16	2.0	0.4	29	59	148	330
Gel Filtration (*)	0.16	47	8.4	2.0	0.32	25	50	156	346

* = In the presence of SDS, reducing agent and renaturation.
ND = Not determined

3. 3. 6 Deglycosylation and Isoelectricfocusing

Glycosylation was thought to be the cause of the polydispersity of the peaks in gel filtration, electrophoresis and isoelectricfocusing. To test this, protein samples from culture filtrates and cell wall-bound fractions were electrophoresed under mild denaturing conditions, renatured *in situ* and activities of the bands were assessed.

The result was that both protein fractions containing xylanase and arabinosidase activities corresponded to a single band of dual activity in the gels (Fig 30a and Fig 30b for arabinosidase and xylanase respectively, of cell wall bound fraction), spanning from 200 to 80 kDa. This band appeared to be glycosylated when stained with PA-Schiff (Fig 30c)

Subsequently, chemical deglycosylation was attempted, initially following the procedure of Edge *et al.* (1981), but despite trying modifications of the method (Naim *et al.*, 1988), enzymatic activities were lost after chemical treatments. Activity was required, to run subsequent IEF gels, therefore deglycosylation was effected enzymatically.

First, denaturing deglycosylation was performed on samples from the affinity-purified complex after gel filtration. Gel filtration fractions showing arabinosidase-xylanase activities and only xylanase activity (see Table 4), were run on SDS-PAGE and the gel stained with silver for glycoproteins (PA-silver, see 3.2.11.3). The result was that the protein fraction showing both activities appeared as one broad band at 116-97 kDa (Fig 30, lane 4) while the protein fractions with only xylanase activity showed different pairs of bands: at 63 and 39 kDa (Fig 30d lane 1) and 56 and 36 kDa (lane 2), calculated from standard curve 1 in appendix 15.

This indicated that the proteins secreted from *S. nodorum* into the medium and that bound to the wheat cell wall (as the cell wall bound protein fraction obtained as indicated in 3.2.2 were used in this experiment) were aggregating through the carbohydrates, at least in certain extent, since several xylanases separated from the complex after deglycosylation when complete denaturation was accomplished beforehand.

Culture filtrates and cell wall-bound protein fractions were used for extensive, non-denatured enzymatic deglycosylation, during the course of which IEF and zymograms for xylanase activity were performed. Since protein aggregates were expected based on previous data, 6 M urea was incorporated into acrylamide gels, and the samples treated with 6 M urea and β-mercaptoethanol before running (see 2.2.6.2.1). Deglycosylation

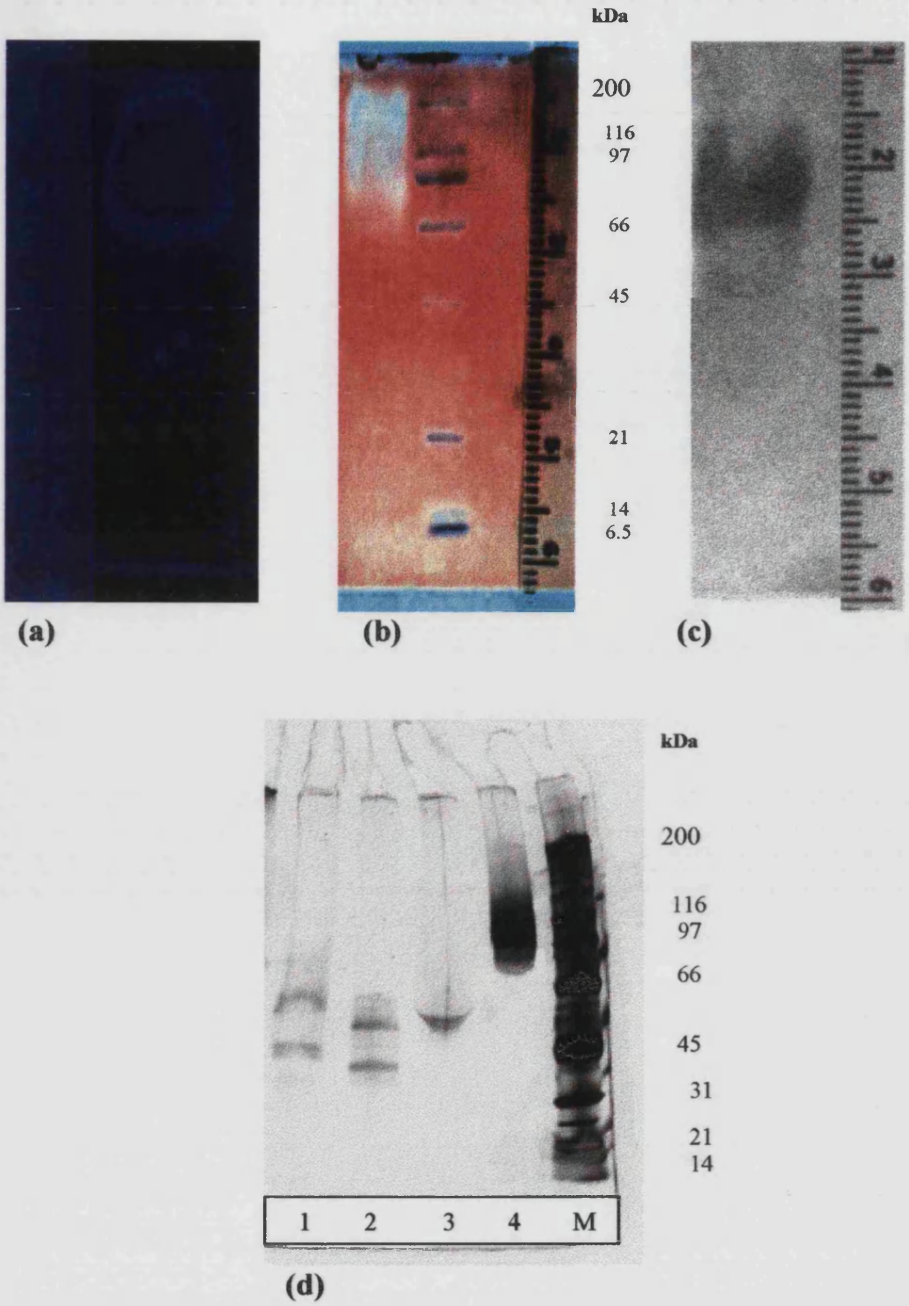


Figure 30 : Mild-denaturing SDS-PAGE of the cell wall bound fraction prior to detection of activity *in situ* [(a) arabinosidase and (b) xylanase], and specific Schiff staining (c); showing dual activity and glycosylation of the protein complex. In (d), SDS-PAGE after denaturing deglycosylation and PA-silver staining of gel filtration fractions that showed only xylanase activity (lanes 1, 2 and 3) and both activities (lane 4).

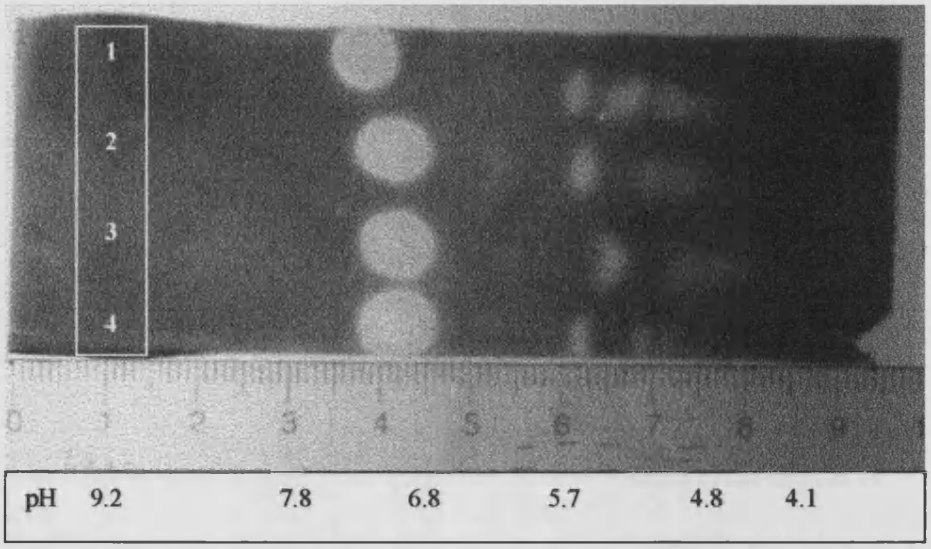
digestion was effected for 3 and 5 days, and samples were run on a gel in the pH range from 3.5 to 9.5.

Both the cell wall bound protein and liquid supernatant showed a discrete number of xylanase isoforms from pI 5.6 to 4.9 (Fig 31a, upper half gel). This was already an improvement since until that moment only smeared zones could be seen for xylanase activity after IEF, even if deglycosylated samples were run in gels without urea. It was also observed that the number of bands was reduced in both samples after 5 days of reaction, from 4 to 3 isoforms (Fig 31a lower half of gel).

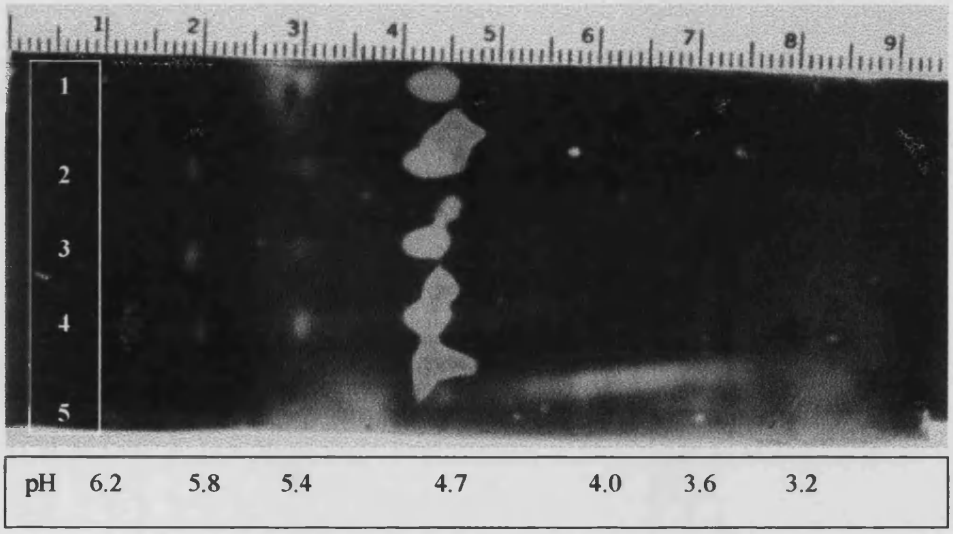
Reduction in isoforms was more evident when deglycosylation was left to continue for another 7 days (in total 12 days of reaction) and the samples run in an urea-containing gel with a narrower pH range (form 2.5 to 7). In this latter case, samples (including a non-deglycosylated control) were treated with 6M urea and β-mercaptoethanol (Fig 31b). After this, two xylanase isoforms with pI's 5.9 and 5.4 were resolved clearly, while the non-deglycosylated sample was resolved as a smeared area spreading towards the anode.

The change in the number of isoforms indicated that the smearing and apparent multiple isoforms was due to, at least in part, the result of microheterogeniety in the glycan moiety of the proteins. Moreover, the migration of the xylanase towards the anode indicated the presence of a negatively charge molecule causing the displacement. Sialic acid was thought to be the molecule responsible for the "abnormal" behaviour of the samples (e.g. non resolution on IEF, and impairment of silver staining).

On the other hand, the arabinosidase-xylanase fraction purified with the crosslinked-lab-arabinan at pH 8.0, showed one isoform of pI 3.8 when overlaid for arabinofuranosidase activity (Fig 32a, lane 1) and of pI 3.6 when overlaid with xylan (Fig 32b). Fractions purified with cross-linked birchwood xylan at pH 4.0 and megazyme arabinan at pH 3.7 showed two arabinosidases with pI's 3.8 and 3.5 (Fig 32a, lanes 2 and 3) and two xylanases of pI's 3.6 and 3.4 (data not shown). The same overlaid gel was stained with silver and showed a single protein band in the crosslinked Lab-arabinan affinity purified

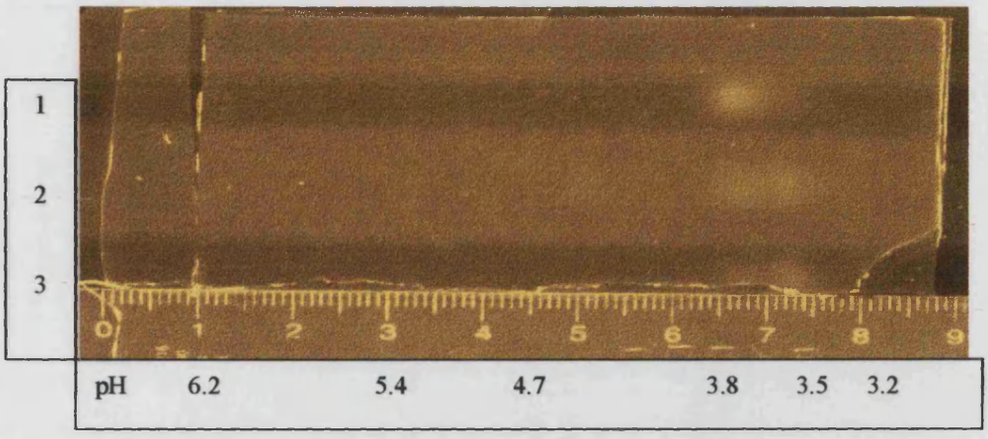


(a) Upper half 3 d digestion, lower half 5 d digestion.

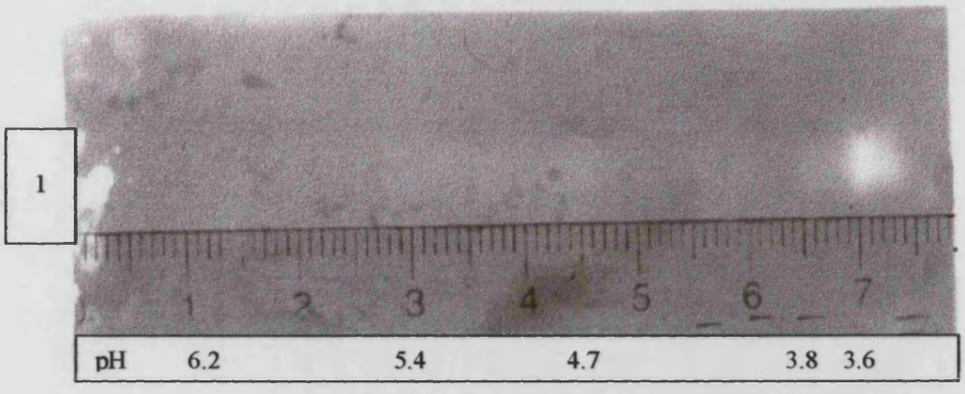


(b) 12 days deglycosylation digestion

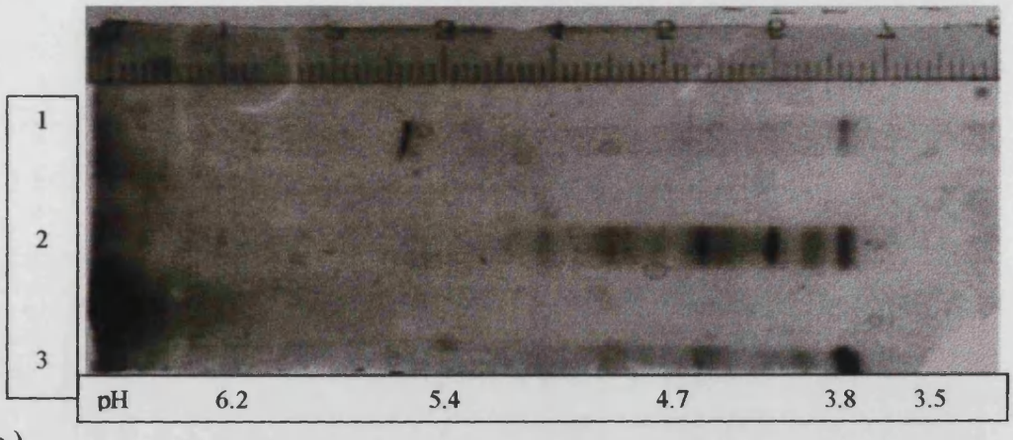
Figure 31 : Isolectricfocusing of cell wall bound fraction (lanes 1 and 3) and liquid filtrate (lanes 2 and 4) after extensive non-denaturing enzymatic deglycosylation and treating the samples with 6 M urea and β-mercaptoethanol. In (a) 6 M urea-gel in the range from 3.5 to 9.5 after 3 days of reaction (upper half gel, lanes 1 and 2) and after 5 days of reaction (lower half gel, lanes 3 and 4). (b) 6 M urea-gel in the pH range from 2 to 7 after 12 days of reaction. The number of bands seems reduced with time of reaction. Also evident is the smearing towards the anode of the non-deglycosylated culture filtrate sample (in lane 5). Clear areas at 4.5cm in the ruler correspond to the application point of samples.



(a)



(b)



(c)

Figure 32 : IEF of affinity purified fractions using cross-linked lab-arabinan at pH 8.0 (lane 1), cross-linked birch wood xylan at pH 4.0 (lane 2) and cross-linked megazyme-arabinan at pH 3.7 (lane 3). After deglycosylation under non-denaturing condition for 24 h, the samples were run and overlaid for (a) arabinosidase and (b) xylanase activities. After substrate gels were used, (c) the IEF gel was stained with the silver protocol of Rabilloud *et al.* (1988).

complex. Several other protein bands were visible in the other two affinity fractions (Fig 32c).

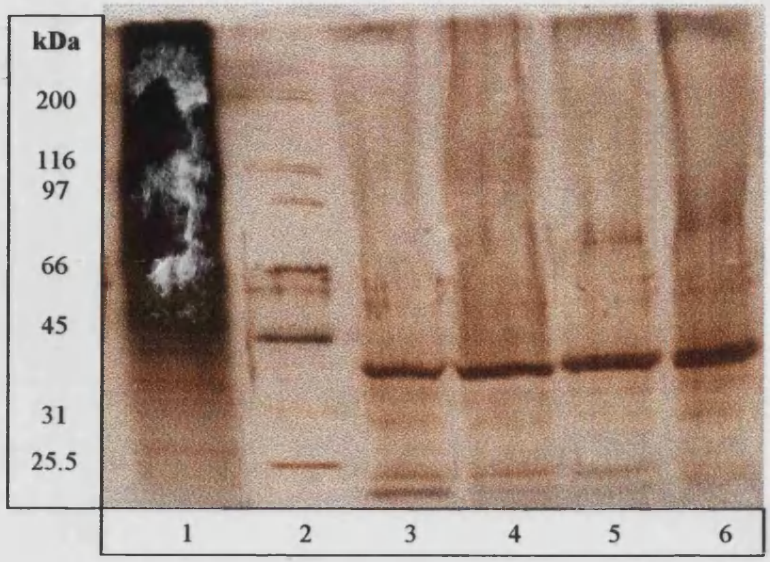
Results indicated that some proteins in the complex could be separated as independent activity bands. Two arabinosidases were clearly separated by IEF in presence of urea from the protein fractions bound to cross-linked birchwood xylan and Megazyme arabinan after deglycosylation. This again implicated the carbohydrates in the conformation of the aggregates. Nevertheless one band (purified from lab-araban at pH 8.0 and present in the other mentioned fractions) appeared to have both xylanase and arabinosidase activity.

3. 3. 7 Blotting and N-terminal sequence

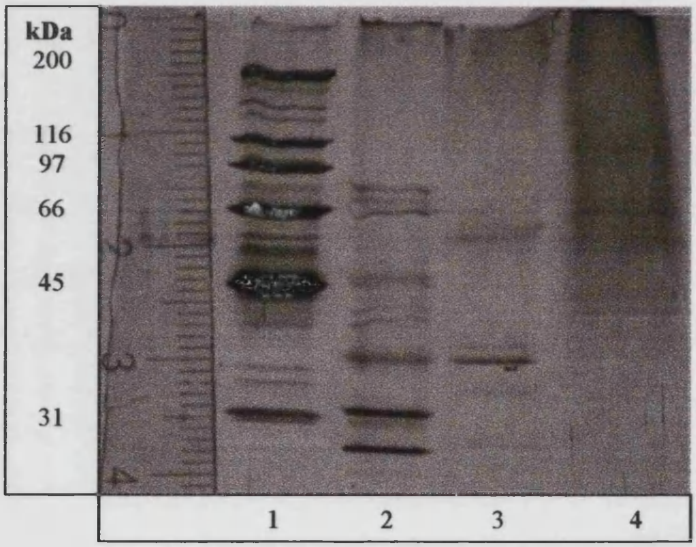
In order obtain sequence from the isolated xylanases and the complex arabinosidase-xylanase, the different groups of pooled fractions from gel filtration (see Table 4) were tested again for both activities, several weeks after they were purified. It was found that arabinosidase was lost on most samples but xylanase activity was still present. An aliquot from each group was deglycosylated enzymatically under complete denaturing conditions and electrophoresis and silver staining was carried out.

The result was that all the groups possessed between 3 to 5 bands piling up together at 35 to 37 kDa (Fig 33a, calculated with the curve 2 in appendix 15). Interestingly, the number of bands clustering in each fraction seemed to increase with the size of the peak eluted from the gel filtration column. Aliquots of each group were taken, mixed and deglycosylated enzymatically, along with a recently purified arabinosidase-xylanse complex. The deglycosylated samples were electrophoresed, blotted and sent to be sequenced. The complex still appeared as a smeared, diffuse band at 70 kDa; the xylanase appeared sharper at 36 kDa (Fig 33b, calculated with the curve 3 in appendix 15).

The bands were blotted onto ProBlott membrane, stained and sent to be sequenced. Results from the N-terminal sequencing are given in Table 6.



(a) Grouped fractions from gel filtration chromatography under mild denaturing conditions. A cluster of 3 to 5 bands piling up very close to each other, appear as a thick band in the picture.



(b) Isolation of putative xylanase-arabinosidase complex from *S. nodorum*

Figure 33 : SDS PAGE of different enzymatically deglycosylated samples, pre-stained with Alcian blue and stained with the silver protocol of Rabilloud *et al.* (1988).
(a) Fractions from gel filtration grouped in order of elution from the column, first eluted (lane 6) to last eluted (lane 3). Culture fluid (lane 1) and MW markers (lane 2) were run along. **(b)** Fractions from 60% ammonium sulphate (lane 2) and affinity purified fraction using cross-linked lab-arabinan at pH 8.0 (lane 3). Culture fluid (lane 4) and MW markers (lane 1) were run along.

Table 6. N-terminal sequence obtained from protein bands at 70 and 36 kDa and with arabinosidase-xylanase and only arabinosidase activities respectively.

(a) Arabinosidase-Xylanase

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S	S	P	V	W	T	S	K	D	V	L	A	N	A	D
		Y	K	E			S	K				T	S	K
							D	G					P	G

(b) Xylanase

1	2	3	4	5	6	7	8	9	10
D	I	N	G	G	G	A	T	L	P
11	12	13	14	15	16	17	18	19	20
Q	K	L	Y	Q	T	S	G	V	L

Arabinosidase gave poor sequence with multiple differences and a variable baseline. When the sequence was submitted to a BLAST search using the NCBI database (www.ncbi.nlm.nih.gov), no similarity was found. Nevertheless, one of the possible combinations of the amino acid sequences corresponded to the deduced amino acid sequence from one of the arabinose cDNA clones isolated after screening a *S. nodorum* cDNA library (Chapter 4). The xylanase sample gave a good sequence with stable base line. Nevertheless, the BLAST search at the NCBI database gave no homology with any reported xylanases but it gave high homology (90%) with the human synovial stimulatory protein, a noncovalent trimer glycosylated complex of 70 kDa. These fragments when blasted to NCBI database showed no identity to any recently described protein sequences. This means that the putative 36 kDa xylanase is the first protein that has homology with human synovial stimulatory protein.

3. 3. 8 Urea Gradient Gels

In order to understand more about the nature of this putative enzymatic complex, the technique described by Creighton (1979) for analysis of structural transitions of the unfolded proteins was used. According to the author, the unfolding of proteins by urea

can be traced by electrophoresis of a band of protein through a slab gel of polyacrylamide in which there is a gradient of urea concentration perpendicular to the direction of electrophoresis, since unfolding is invariably manifested by marked reduction of mobility, presumably due to molecular sieving of the expanded polypeptide chain by the polyacrylamide gel.

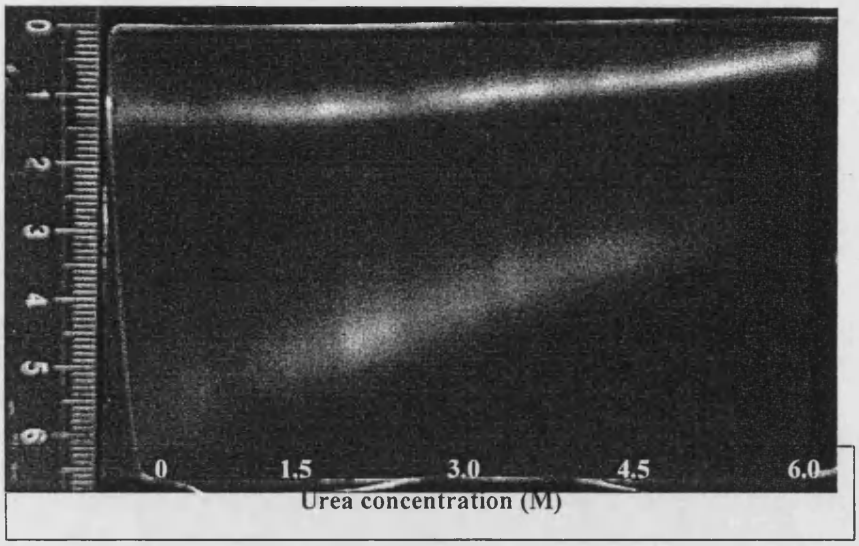
Since the renaturation and restoration of activity of arabinosidase and xylanase of *S. nodorum* could be achieved, the approach used was the running of purified samples on urea gradient gels, comparison with running in the presence of denaturing and reducing agents, and analysis of the activity band patterns to confirm the presence of a bifunctional enzyme and/or aggregates of enzymes.

The urea gradient pattern of the native arabinosidase is presented in Fig 34a lower part of the gel, and the corresponding xylanase activity in Fig 34b lower part of the gel. The electrophoretic mobility of the arabinosidase activity decreased with increased urea concentrations, but it was a gradual change, without an abrupt transition. This means that as concentration of urea increased gradually, there were intermediate mobilities due to a presumed equilibrium between the two states (folding and unfolding); the resultant continuous band then, reflected the equilibrium proportions of the two species. Though the activity band was continuous, it was not sharp, which may indicate slow equilibration of the folded and unfolded states.

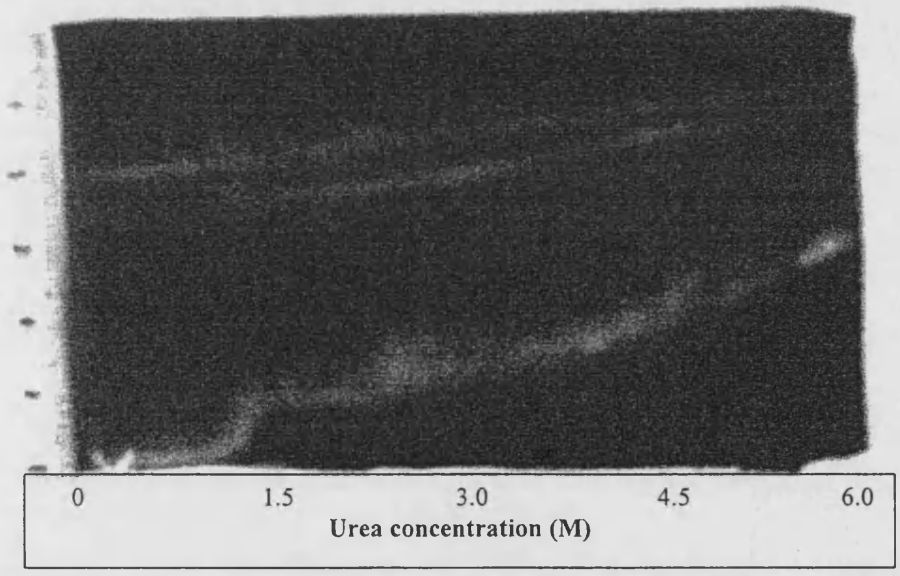
Low concentrations of urea produced no change in the electrophoretic mobility of the xylanase activity band until a concentration of around 1.5M, where a relatively sharp change in mobility occurred, indicative of the onset of unfolding of the protein. With increasing concentrations of urea above this inflection point, the mobility of the activity band decreased steadily indicating that not all structural changes occurred at once, but in a manner similar to the arabinosidase pattern. Moreover, arabinosidase and xylanase activity bands seemed to overlap or at least they were very close one to the other.

When the same sample was treated with SDS and β-mercaptoethanol, the reduced and denatured arabinosidase band migrated more slowly than the folded one but gave no indication of any further unfolding induced by urea, which is in agreement to it being already completely unfolded by the detergent and the reducing agent (Fig 34a, upper half of the gel).

For the reduced and denatured xylanase, a second discontinuous xylanase activity band appeared associated with unfolding at around 1.5M (Fig 34b, upper half of the gel). These results are consistent with a simple dissociation of an oligomeric protein into subunits, with variation in molecular conformation occurring at urea concentrations more than the transition region; being necessary the presence of SDS and a reducing agent were necessary to make them evident as separate entities. A summary of the experiments described in this chapter is presented in Table 7.



(a)



(b)

Figure 34 : Urea gradient electrophoresis at 900 V and 8 mA of the putative purified arabinosidase-xylanase complex. First, protein sample alone was dissolve in sample buffer (0.06 M Tris-HCl, pH 6.8), applied and run for 7 h (lower part of gel); after that, more protein was dissolve in sample buffer plus 1% SDS and 280 mM β-mercaptoethanol, applied and run for further 2h 30 min (upper part of gel). This was followed by renaturation of the gel and overlays for arabinosidase (a) and for xylanase (b), to reveal the enzymatic traces of the protein complex. Electrophoresis towards the cathode was at 10 °C. The resolving gel buffer was 0.375 M Tris-HCl pH 8.8. The linear urea gradient was from 0 M to 6 M.

Table 7. Summary of attempted separation and purification of arabinosidase and xylanase and evidence for glycosylation and complex formation

EXPERIMENTS	RESULTS
1. Ammonium Sulfate Precipitation	Arabinosidase and xylanase activities could be purified up to 1.9 and 4.7 times with 65% (w/w) ammonium sulphate saturation at 4 °C
2. Ion exchange and hydroxyapatite chromatography and IEF	Based on preparative IEF, xylanase isoforms in the culture filtrates were expected to span a broad pH region. Nevertheless, the enzyme showed unpredicted charge behaviour. In hydroxylapatite chromatography, protein fractions behaved as polydisperse in respect to charge and hydrophobicity. This, together with results from analytical IEF, was evidence for protein glycosylation.
3. Affinity chromatography (AFC) and SDS-PAGE	Both arabinosidase and xylanase bound together to the crosslinked-substrates xylan and arabinan. Binding was dependent of pH, giving different number of protein bands in SDS-PAGE. Selection of most appropriate affinity supports and pH of binding was achieved. SDS-PAGE and specific staining gave further evidence for protein glycosylation.
4. Gel filtration chromatography (GFC) and SDS-PAGE	Protein fractions from AFC showed microheterogeneity in gel filtration chromatograms. With GFC under mild denaturing conditions it was possible to separate AFC fractions into different peaks: the biggest with both arabinosidase-xylanase activity and one or two, smaller ones, with only xylanase activity. GFC also removed tightly bound carbohydrates, which were about 30% (w/w) of total carbohydrate content. Glycan moiety in protein samples after GFC was around 45% (w/w). These results together with SDS-PAGE of GFC fractions revealed that carbohydrates, to some extent, facilitated aggregation.
5. Enzymatic non-denaturing deglycosylation and analytical IEF in the presence of urea	After removal of sugar moiety and sialic acid from diverse samples, it was possible to determined xylanase isoforms in analytical IEF in the presence of urea. Also different isoforms were resolved for affinity fractions. This indicated than more than protein aggregates the existence of a multimeric-multifunctional enzyme was possible. Sugar and sialic acids contents in the putative purified complex were around 47% (w/w) of mannose equivalents and 8% (w/w) of NANA equivalents respectively.
6. Urea gradient gels	Folding and unfolding of the putative purified complex revealed one polypeptide chain with both arabinosidase-xylanase activities, non separable after reduction of SH-bounds and SDS binding, and another subunit with xylanase activity that appeared in the unfolded state after reduction and SDS binding.

3. 4 Discussion

Two *Stagonospora nodorum* CWDE, namely arabinosidase and xylanase, were chosen for purification from culture fluids with wheat cell walls as the carbon source. During the attempted purification both enzymes appeared to be present in the same fractions with many different separation methods. Moreover, earlier time course experiments in which different CWDE produced in culture fluids and in infected tissues were measured and compared, showed that xylanase, arabinosidase and xylosidase displayed the same pattern, possibly revealing enzyme association or similar regulation. These and other lines of evidence suggested the existence of an enzymatic complex or xylanosome-type enzymatic multiprotein in *S. nodorum.*

Both bacteria and fungi have been reported to produce "xylanosome" multi-enzyme complexes, as in the case of the cellulase-xylanase system of *Clostridium papyrosolvens* (Pohlschroder *et al.*, 1994), the xylanase-arabinosidase of *Pseudomonas fluorescens* (Kellett *et al.*, 1990), the multi-subunit protein aggregate of xylanases of *Butyrivibrio fibrisolvens* H17c (Lin and Thomson, 1991); and the xylanase multiprotein complexes of the fungi *Piromyces equi* (Fillingham *et al.*, 1999), *Penicillium canescens* (Gaspar *et al.*, 1997) and *Neocallimastrix frontalis* (Wilson and Wood, 1992)

Clostridium papyrosolvens for example, is capable of hydrolysing crystalline cellulose and xylan by secreting a high-molecular-weight enzyme system of at least seven protein complexes denominated by the authors as multicomplex fraction. The multicomplex fraction was resolved into other multiprotein peaks by means of anion-exchange chromatography. The relative heights of the protein peaks varied from experiment to experiment, and additional peaks were observed in some experiments. All fractions contained several glycosylated proteins and differed in properties from each other; two of

them having only xylanase activity (Pohlschroder *et al.*, 1994; Cavendon *et al.*, 1990a; Cavendon *et al.*, 1990b).

Enzymes secreted by *Bacillus sp.* and the ruminal fungus *Neocallimastrix frontalis* as a multiprotein complexes contained different enzymatic activities, among them endoxylanses with diverse characteristics. In the case of the bacterial enzymes, they appeared to be glycosylated bifunctional xylanases, one with activity over xylan and cellulose and the other with xylanase and xylosidase activity (Jaishree and Varma, 1992). *N. frontalis* showed two xylanases, one glycosylated and the other non-glycosylated, but both exhibited xylanase and cellulase activities (Gomez de Segura and Fevre, 1993). Xylanase and cellulase activities have been reported for other fungi such as *T. harziamun* (Tan *et al.*, 1985), *Aspergillus kawachii* (Ito *et al.*, 1992) and *Neurospora crassa* (Deshpande *et al.*, 1986).

According to these studies and others, major indication of the existence of a multiprotein complex is its stability; that is the resistance of the complex to be separated through different separation techniques and that the complex can adsorb reversibly to insoluble substrates and hydrolyse them (Deutscher, 1990). The putative complex in *S. nodorum* showed that many attempts to separate xylanase and arabinosidase were unsuccessful. Different purification techniques included ion exchange chromatography, preparative IEF, hydroxylapatite chromatography and gel filtration chromatography under mild denaturing conditions. The complex bound reversibly to birch wood and oat spelt xylans and also to wheat cell walls. In ion exchange chromatography the proteins bound well to the supports but behaved as a unit, i.e. either bound or unbound together in the same peaks. In addition most of xylanase and arabinosidase activities failed to change to cationic form in low pH solutions and remained negatively charged.

The possibility that different proteins were secreted by *Stagonospora nodorum* along with polysaccharides of anionic nature was thought to be the cause of aggregation of proteins and the behaviour of these enzymes in ion exchange chromatography and IEF. Indeed, gel

filtration under denaturing conditions, SDS-PAGE and specific PA-Schiff staining demonstrated that there was polysaccharide firmly bound to the proteins and causing them to aggregate but also that the proteins were glycosylated, this contradicted with the failure to bind to the concanavalin A lectin used as affinity support. Explanation for this is that not all glycoproteins bind to all lectins used for affinity chromatography. Different lectins bind different types of carbohydrates and in determined structures. A screening for selection of a suitable lectin for affinity chromatography is therefore usually required (Carlsson, 1993).

Glycosylation was completely in agreement with the complicated, smeared patterns of arabinosidase and especially xylanase in analytical IEF and gel electrophoresis. Pure proteins are generally monodisperse with respect to physical properties such as molecular weight or charge (i.e. all molecules in the population are identical in size or in charge). Glycoproteins however, often show paucidispersity (i.e. few molecular species that form part of a single statistical population) or polydispersity (i.e. larger number of molecules of a single statistical population), in their physical properties. For instance considering molecular weight, polydispersity gives broad bands on SDS-polyacrylamide gel electrophoresis and broad peaks in gel filtration elution profiles. Similarly, polydispersity in charge gives broad bands in IEF and poorly resolved peaks in ion exchange chromatography (Beeley, 1985; Andrews, 1981)

Glycoproteins with low carbohydrate content (<5% by weight) behave more or less in the same way as for non-glycosylated proteins. However, this was not the case for the culture fluid proteins of S. *nodorum*, whose complex performance and poor reproducibility throughout the different experiments indicated that large amounts of carbohydrate were present, which gave rise to difficulties probably because of polydispersity, molecular interactions and high charge density.

These difficulties in resolving clearly electrophoretic and chromatography techniques also indicated complexity of the glycan moiety since the variety, intricacy and heterogeneity of

the glycan chains in glycoproteins are associated with structural and molecular characteristics, such as determination of size, shape, pI, molecular weight, etc. (Durchschlag and Jaenicke, 1997).

During this study, non reproducibility of results, in particular in gel filtration chromatography and electrophoretic techniques (SDS-PAGE and IEF), became a major problem in establishing criteria for assessing the homogeneity of protein samples, adding to the multiple activities they showed. Glycoproteins containing more than few percentage of carbohydrates can give serious errors in molecular weight analysis by gel filtration or polyacrylamide gel electrophoresis. Gel filtration columns were calibrated with proteins of known molecular weight to obtain standard curve of elution volume (Ve) against logarithm of molecular weight (Log M). While data of most globular proteins fall on this curve, the elution volumes of glycosylated proteins of known molecular weight generally lie below the calibration curve. The reason for the anomalous behaviour of glycoproteins is that their asymmetric, branched structure gives them an apparent Stoke's radius greater than that of a globular protein (Andrews, 1981).

In addition, there was some type of ionic interactions of the proteins, noticeably the smearing towards the anode and peculiar behaviour in ion exchange chromatography of xylanases. Frequently these interactions arise from the side chains of glycoproteins that generate diverse charge in solution, affecting significantly the IEF. It is mostly variation in sialic acid content between molecules (polydispersity in respect to sialic acids), which may lead to anomalous IEF patterns (Durchschlag and Jaenicke, 1997).

Sialic acids, conferring strong anion character to these proteins were evidenced by the fact that they did not stain very well with the normal Coomassie brilliant blue or silver staining procedures; giving negative bands against the brownish background of silver stained gels, a typical indications of highly anionic proteins (Carlsson, 1993). Whereas, the cationic dye Alcian blue, which has been widely used for staining acid polysaccharides and glycosaminoglycans (Powel *et al.*, 1982), revealed clearly the protein bands.

In another experiment in which 2% (w/v) phosphotungstic acid was used in fixation before staining of SDS-PAGE, gels showed a blackish smear not evident in gels with normal staining (data not shown) (e.g. Fig 24). Very anionic proteins with abundant sialic acid are resistance to precipitation with high concentrations of ethanol, ammonium sulphate or trichloroacetic acid, while phosphotungstic acid is an excellent precipitant and is used for a rapid silver staining of IEF agarose gels for anionic proteins (Lebrun-Fourcy *et al.*, 1996). The presence of sialic acids explain the appearances of the blackish smears in Fig 24 and the solubility of the putative complex arabinosidase-xylanase complex at high concentration of ammonium sulphate (60% saturation) while other proteins of the liquid fluid precipitated; also the impairment of the silver staining and the behaviour of the samples in PAGE electrophoresis, IEF and ion exchange chromatography.

Removal of glycan moieties and sialic acid was obtained with enzymatic deglycosylation. These required the protein to be completely denatured (treatment with SDS at high temperature followed by saturation with a non-ionic detergent), since some residues are extremely resistant due to steric hindrance (Gianazza, 1995). However, determination of IEF and determination of multiple activities for the same polypeptide chain required the activity of enzymes. So far, removal of glycan moieties and sialic acid under non-denaturing conditions reduced xylanase microheterogeneity, and it was assumed that only two xylanase isoforms were present in the culture fluids of *Stagonospora nodorum*. Whether other real isoforms were present and were degraded during the long deglycosylation treatment has to be confirmed. This uncertainty is due to the kinetics of neuraminidase treatment that resolve intermediate bands corresponding to the stepwise removal of sialic acid in a manner that the number of bands matches the number of such residues (Gianazza, 1995).

The content of sialic acid was determined after enzymatic release in the purified samples and the cell wall bound protein fraction using sialidase A (Table 4). It was found that the liberated and lyophilised sialic acid was not detectable by the TBA assay for sialic acids

unless treatment with NaOH was effected previously. This is an indication of *O*-acetylation occurring at carbon 7, 8 or 9 (Beeley, 1995; Klein and Roussel, 1998).

Interestingly, in affinity-purified fractions arabinosidase activity, that was very stable for long periods of time even after treatment with SDS at 60 °C, was reduced drastically with progression of the removal of sialic acid (up to 70%), even though sialic acid was removed in the presence of an antiprotease cocktail. Conversely, xylanase activity was stable after a long sialidase treatment. Two possibilities can describe this phenomenon. Perhaps protease activity was not responsible for the loss of the arabinosidase activity in the affinity-purified fraction in the early development of the sialidase treatment, but sialic acid gave stability to the complex. Linked glycans can affect protein structure facilitating the protein folding process, stabilising the mature protein and assisting the assembly of oligomeric complexes to such extent that its removal can alter protein function (Imperiali and O'Connor, 1999). Although such roles have not been attributed specifically to the terminal sialic acids in the glycan moieties, it can be speculated that sialic acids may play an important function in the carbohydrate-mediated stability as in any other carbohydrate-dependent characteristic, due to its physico-chemical properties, mainly electronegativity, hydrophilicity and relatively large size of hydrated molecules.

In general sialic acids are thought to be one of the chief surface reactive components in fungi, particularly contributing to pathogenicity as inhibitors of phagocytosis in those invading mammalian tissues, who are the foremost fungi studied. For example comparison of infective yeast and saprophytic hyphae of *Sporothrix schenckii* showed that the infective forms have double acidic layer rich in sialic acids, whereas hyphae have a single acidic layer. In this species the distribution of anionic sites on the infective yeast forms and external cell wall layer sloughed off from these cells was tentatively associated with fungal virulence of the infective yeast. Released surface antigen in the form of aggregated membranous bodies could interact with antibodies and other elements of the immune system leaving the infective fungus unchallenged to proliferate and invade tissues (Oda *et al.*, 1983). The same methodology was used to evaluate the relevance of silaic acids as

contributors to the negative charge of the fungus *Fonsecaea pedrosoi* (Souza *et al.*, 1986), and similar results were obtained.

Oda *et al.* (1983) and Rodrigues *et al.* (1997) gave some evidence supporting the hypothesis that sialic acids may contribute to the pathogenicity of fungi and that Neu5Ac is an effective inhibitor of phagocytosis when demonstrated that sialic acid residues expressed at the cell surface of *S. schenckii* and *Cryptococcus neoformans* protected fungal cells from phagocytosis by resident mouse peritoneal macrophages. Enzymatic removal of sialic acids from the external layers of *C. neoformans* and *S. schenckii* envelopes renders yeast cells more susceptible to phagocytosis.

Very little is actually known about the specific biological role of this molecule especially in plant-pathogen interactions (Alviano *et al.*, 1999). To the author's knowledge this is only the second report of sialic acid in any phytopathogenic fungus.

A second possibility is that protease activity was present in the samples and the sialic acid was the main molecule conferring protection against proteolysis. This proteolytic activity would also explain the slow degradation of arabinosidase activity in the grouped gel filtration fractions in which glycosylation (and mainly sialic acid) delayed protein degradation, until the bands piling up very close to each other and seen as thick band in Fig 33 were obtained, and when no further proteolysis was possible. At this respect, *O*-acetylated sialic acids have been found responsible for protection against proteolysis (Klein and Roussel, 1998). Also, active proteases of *S. nodorum* are produced under these cultures conditions (Carlile *et al.*, 2000) and are expected to be present in the samples.

Also assessed were the structural modifications of the affinity-purified enzymes, implying non-covalent binding (as for the holo foms of carrier proteins and prosthetic enzymes), the covalent binding (S-S bridges) and non-covalent interactions between subunits with oligomeric structures (multienzymatic complexes). These modifications constitute another characteristic that can be analysed by electrophoretic techniques.

IEF under denaturing conditions (high concentration of urea) give proteins a random coil spatial arrangement that implies no restrictions on relative movement between its different segments. This way, each singular segment can exhibit its singular pI depending on the amino acid composition (Gianazza, 1995). Using this approach, no more than two isoforms were resolved for xylanase and for arabinosidase in the affinity-purified samples when binding occurred at different pHs. This again is in agreement with the reduction of microheterogeneity for the xylanase activity in the culture fluid and cell wall-bound fractions; however the presence of charged molecules, in particular sialic acids in glycoproteins, impairs the resolution of the pI since the pK of sialic acid (which around 3) add to the pK of other specific groups producing shifts in the tritiation and altering the pI of the whole macromolecule (Gianazza, 1995).

Electrophoresis in urea gradient gel allows a protein composed of several sub-units to undergo a series of transitional arrangements according to the different concentrations of urea. If other denaturing agents such as SDS or β-mercaptoethanol are added to the proteins the separation of subunits may be completed e.g. all subunits bound by SH bridges or other interactions will separate and just covalently bound polypeptide chain remain together (Creighton, 1979; Gianazza *et al.*, 1997; Kresl *et al.*, 1998).

Using this analysis, it was found that when the affinity chromatography purified complex was run under a gradient 0 to 6 M urea, one xylanase activity and one arabinosidase activity run along in what seems to be a single polypeptide chain with double activity, more probably this was in two different domains, that is, a putative bifunctional arabinosidase-xylanase. Another subunit with xylanase activity was detected, separating from the complex after reduction with β-mercaptoethanol and when the complex was exposed to SDS and a concentration of around 1.5M of urea.

Multiple banding under the above mentioned conditions has been interpreted by several research groups as a result of the presence of disassembled oligomers and applied to

investigate effects on protein stability brought about by mutation (Carra and Privalov, 1995); posttranslational processing (Powel and Pain, 1992) and chemical modifications (Sun *et al.*, 1992). Following this interpretation, the behaviour of the affinity-purified arabinosidase-xylanase in the presence of additives such as urea, SDS and thiol *versus* disulfide states of the protein and at the pH of experimental conditions, indicated so far three domains (two different xylanases and an arabinosidase), with the possibility that a xylosidase make also part of the complex.

The number of bands in the clusters of the SDS-PAGE of grouped fractions from gel filtration chromatography under mild denaturing conditions in Fig 33a, in which the number of piling up bands seems to be increased with increased size of the eluted peaks, may be indicative of the association of different or repetitive subunits, perhaps different xylanases; or be the result of paucidispersity after deglycosylation. The same could be said about the diffuse band in the SDS-PAGE of the arabinosidase-xyalanse in Fig 33b.

Results from the sequence of both the putative xylanase and arabinosidase-xylanase subunits will be discussed in Chapter 4 along with results from DNA sequences.

Though the catalytic domains of the putative affinity-purified arabinosidase-xylanase complex appeared as single diffused bands in the SDS-PAGE in Fig 33b and in the lower parts of the urea gradient gel of Fig 34a and 34b, does not imply intradomain inflexibility. This means that different interactions of the three domains (detected in the urea gradient gel when SDS and β-mercaptoethanol were used) can take place at, for example, different pH's, as pH certainly change in environmental conditions *in vivo*, and given that the mentioned variable was not considered in the gel gradient experiments during this study. In this respect, Ginazza *et al.* (1997) found that differences in genetic variants of human serum albumin could be detected as protein instability of the different protein domains and in consequence multiple banding, when the protein was electrophoresed in various urea gradient gels at a different pHs and also across a wide range of pH gradient gel set by focused carrier ampholytes.

In summary, results from different chromatogramatographic and electrophoretic techniques of the culture fluid and the wheat cell wall-bound proteins can be taken as evidence of the existence of a complex, that separated with affinity chromatagraphy in cross-linked lab-arabinan at pH 8.0 was composed of a bigger protein with arabinosidase–xylanase activity and other smaller proteins with xylanase activities. Different bands binding to the cross-linked supports at a different pH's may reflect the pH-dependence interaction of several domains within the complex. Peaks from gel filtration chromatography under mild denaturing conditions were in agreement with this result. Polydispersity in respect to size and charge, the results from specific staining and from desialyation and deglycosylation of the putative enzymatic complex, suggested that it was glycosylated and contained modified sialic acid; which may be *o*-acetylated at 7, 8 or 9 positions.

CHAPTER 4 A DNA BASED APPROACH FOR CHARACTERISATION OF THE DIFFERENT XYLANASES AND ARABINOSIDASES

4. 1 Introduction

Enzymatic hydrolysis of the xylans is effected by a consortium of microbial enzyme, including endo-β-1,4-xylanses, β-xylosidase and a series of enzymes that cleave side chain sugars (glycosidases) or remove acetyl groups from the xylan backbone (Millward-Sadler *et al.*, 1995; Gilbert and Hazlewood, 1993). While considerable effort has been focused on studying the endo-β-1,4-xylanases and β-xylosidases, there is much more limited information about the enzymes and their genes responsible for the cleavage of side groups even though there is much evidence for these activities in the complete and efficient degradation of the heterogeneous polymer (Merino-Trigo *et al.*, 1999). More genetic information is actually needed, in particular for enzymes releasing L-arabinose from *Gramineae* cell walls, like that described by Vincent *et al.* (1997) where a purified α-L-arabinofuranosidase from *Streptomyces lividans* had specific arabinofuranose-debranching activity on xylans from rye, wheat and oats, to which it bound specifically and acted synergistically with *S. lividans* xylanases. It liberated arabinose and after prolonged incubation it released xylan from small arabinoxylo-oligosaccharides. Interestingly the genetic study demonstrated that the *abfB* gene is divergently transcribed from a previously identified xylanase gene (*xlnA*), and that the N-terminus of the transcribed AbfB arose from gene duplication of the C-terminus of XlnA located in the upstream vicinity.

Many cellulases consist of two distinct domains comprising a catalytic domain (CD) and the non-catalytic binding domain (generally known as CBDs), both domains are linked by a hinge region rich in hydroxylamino acid / proline rich sequences. In many hemicellulases, cellulose-binding domains or more recently described xylan-binding domains, very similar to CBDs, are quite commonly found in most cellulases, and

located in the noncatalytic regions of the enzymes. The role of the XBDs located in hemicellulases is unclear, although it is thought that they bring the hemicellulase into contact with the plant cell wall. Xylan-binding domains (XBDs) were reported to be present in genes isolated from *Cellulomonas fimi*, *Thermomonospora fusca* and *Streptomyces lividans* (Irwin *et al.*, 1994; Black *et al.*, 1995; Vincent *et al.*, 1997; Dupont *et al.*, 1998). The deduced amino acid sequences of these XBDs show some similarities to those of bacterial CBDs.

Septoria nodorum produces an enzymatic system that is able to degrade wheat cell wall xylan and this system is able to adsorb to insoluble xylans, cross-linked xylans, cross-linked arabinans and isolated wheat cell wall (as seen in previous chapters). Although the role of these enzymes in pathogenesis is not fully clear yet, new approaches may help in the study of this system. In that context, the complexity of host-pathogen interactions, particularly in the framework of changing cellular and tissue environments encountered by invading pathogens, means that conventional physiological and biochemical approaches can only give, at best, equivocal answers to the understanding of the mechanisms of microbial pathogenesis. With the development over the past two decades of molecular genetics comprising a variety of screening and selection methods, the identification and characterisation of microbial gene products that are expressed during infection along with disruption or deletion of the corresponding genes, has become a become a feasible approach, and is contributing enormously to the study of the molecular mechanisms of pathogenesis (Hensel and Holden, 1996)

The biochemistry and molecular genetics of the xylanase systems of some fungi and bacteria have been exhaustively studied in the context of numerous applications of these enzymes in industrial fields, mainly food and feed industries, paper pulp treatment, and removal of residues from agricultural and forestry activities. These studies have provided much impetus and critical background information on the structure and function of these enzymes, which should facilitate the study of plant pathogenesis.

4. 2 Materials and Methods

4. 2. 1 Biological Material

E. coli strains XL1-Blue MRF'and SOLR (Stratagene Inc.) were grown on LB liquid culture medium (appendix 16A) at 37 °C and under agitation at 200 rpm . When required, NZY medium was used instead (appendix 16B). Stock solution of antibiotics were prepared and sterilised by filtration, and then appropriate volumes of stocks were added to the medium after cooling to around 50 °C. When liquid cultures were used, 50-ml Sterilin tubes were filled with 10 ml medium and incubated at 37 °C with shaking at 200 rpm for not longer than 12 h.

Bacteria carrying the isolated xylanase and arabinosidase plasmids were preserved at -80 °C in a filter-sterilized solution of LB broth and glycerol 25 % (v/v). Plasmid pBluescript KS (Stratagene Inc) and lambda vectors Uni-Zap XR (Statagene Inc) and ExAssist helper phage (Statagene Inc) were used in this study. *Stagonospora nodorum* isolates BS 471, 171, Ln 97 and LAW were grown on CzV_8Cs liquid medium as described on section 2.2.1.2. for fungal DNA extraction.

4. 2. 2 PCR Approach for Designing Arabinosidase and Xylanase Probes for Screening the cDNA Library

A collection of amino acid and nucleotide sequences of arabinosidases and xylanases from fungi and bacteria were aligned using the Multiple Sequence Aligment Clustal W programme (biology work bench at http://workbench.sdsc.edu). Three regions of amino acid and nucleotide sequences with high similarity were identified. Oligonucleotides were designed based on these regions and employed as primers in polymerase chain reaction (PCR), to amplify DNA fragments from genomic DNA of five isolates of *S. nodorum.* One of the oligonucleotides for each enzyme was used as an internal primer for nested-PCR in order to test the fragments.

This resulted in the amplification of products of expected sizes, which were then excised from the gels and sequenced using the internal oligo as a primer. Sequence data was submitted to a blastx search using the NCBI database (www.ncbi.nlm.nih.gov). Both PCR products showed strong homology to fungal and bacterial xylanases and arabinosidases respectively. These PCR-amplified DNA products were used later as probes for screening the cDNA library as described in section 4.2.6.

4. 2. 3 Isolation of Fungal DNA

The procedure by Fulton *et al.* (1995) was followed. Fresh mycelium from liquid cultures was rinsed with distilled water and ground in liquid nitrogen with a pestle and mortar. To 100 mg of powdered mycelium, 1ml of extraction buffer was added into a 1.5 ml propylene tube. Extraction buffer was prepared immediately before use, consisting of 2.5 volumes of solution A [0.35M sorbitol (Sigma), 0.1M tris pH 7.5, 5mM EDTA(BDH)] and solution B (0.2 M tris pH 7.5, 50 mM EDTA, 2 M NaCl, 2% CTAB); plus 1 volume solution C (5% Sarcosyl, Sigma). Solutions A and B were kept at room temperature. Solution C was prepared immediately before use.

Ground hyphae and extraction buffer were shaken and incubated at 65 °C for 60 min after which 0.5 ml of a solution of chloroform:isoamylalcohol (24:1) was added and mixed thoroughly. The tube was centrifuged at 10000 xg for 15 min. The supernatant was removed and 0.6 ml isopropanol added to precipitate the DNA. After centrifugation at top speed speed (10000 xg) for 15 min and decanting, the precipitated DNA was washed with 70% (V/V) ethanol and dissolved in TE buffer.

4. 2. 4 Electrophoresis and Manipulation of DNA

Gels of different agarose concentrations were used depending on the expected DNA size in order to give the best resolution of DNA bands (see appendix 17). Gels were prepared in 0.5X TAE (Tris-acetate 40mM, EDTA 2 mM), by dissolving and melting

by several 30-second pulses in a microwave oven. Gel slabs were poured to a thickness of 0.5 to 1 cm with a gel comb in place to give sample wells of appropriate dimension. Running buffer used was 0.5X TAE. DNA samples were prepared by dilution of one sample volume with 1/5 that volume of loading buffer (consisting of 0.25% (w/v) bromophenol blue and 30 % (v/v) glycerol in MilliQ water. A volume of 5µl of Hyper ladder I (Bioline) ranging from 200 to 10,000 bp, was used as a molecular weight marker and for quantification of DNA on gels.

Sample DNA concentration was estimated by comparison of band intensity with that of the markers, according to the manufacturer's chart. Electrophoresis was carried out at 4-8 V/cm for 1 to 1.5 hours. After electrophoresis was terminated, gels were immersed in ethidium bromide solution (1µg / ml) for 10 to 15 min and then DNA bands were visualised and documented under UV light using a UVP white/UV transilluminator and digital graphics printer.

4. 2. 4. 1 Recovery of DNA from Agarose Gels

PCR products or DNA bands of interest were recovered from agarose gels using either the Pharmacia Biotech "Sephaglass Band Prep" kit or the Qiagen "QIAEX II Gel Extraction " kit, according to the instructions of the manufacturers.

4. 2. 4. 2 DNA Precipitation and Concentration

When is was necessary, ethanol precipitation of DNA was carried out by mixing 0.1 volumes of 3M sodium acetate (or 0.25 volumes of 10M ammonium acetate) and 2.5 volumes of 95% ethanol. This mixture was then added to the DNA sample and tubes were placed on ice for 15-20 minutes. After centrifugation at top speed (10,000 xg) for 15 minutes in a bench-top centrifuge the supernatant was discarded and the pellet was re-suspended in 1ml of ice cold 70% ethanol. The tube was centrifuged as previously, the supernatant discarded and the pellet allowed to air dry for at least 10 minutes before re-suspension in a suitable volume of sterile MilliQ water.

4. 2. 4. 3 Restriction Digestion of DNA and Southern Blotting

DNA solutions of known concentration were mixed with restriction enzymes in appropriate buffer according to the specifications of the manufacturer (Kramel Biotechnologies, New England Biolabs, Bioline or Advanced Biotechnologies). Restriction digests were set up according to the manufacturer's instructions, taking care to ensure that the volume of restriction enzyme did not exceed 1/10 the final restriction volume, in order to avoid inhibition of enzyme activity by glycerol in which commercial enzyme preparations are stored. A typical reaction mixture is shown in appendix 17C. Reactions were incubated at 37 °C for 1 to 2 hours after which reactions were stopped by transferring the tubes into an ice bath.

Genomic DNA restriction digest samples were electrophoresed on a 0.8% agarose gel prepared on 0.5X TBE (Tris-borate 89mM, EDTA 2 mM, appendix 17B) and run with the same buffer at 1-2 v/cm overnight. The gel was then immersed in depurinating solution (0.25M HCl) and gently shaken for 15 minutes. The solution was poured off and the gel rinsed with distilled water and placed into denaturing solution (0.5 M NaOH, 1.5M NaCl) with shaking for 30 minutes. The gel was rinsed as previously and immersed in neutralising solution (1.5M Tris-HCl pH 7.0, NaCl 1.5M) with shaking for 30 minutes. Southern blot apparatus was then set up, which consisted of a glass tray containing 5-10mm depth of 10X SSC (appendix 18c). A plastic gel tray was placed inverted in the liquid then it was covered with 2 sheets of Whatman 3mm paper soaked in 10X SSC in such a way that ends of the paper were immersed in liquid forming a wick. Care was taken to remove any air bubbles by gently rolling with a clean plastic 10ml pipette. The gel was then placed inverted on the wet paper bridge and overlaid with a piece of nylon membrane (Hybond N+) the same size as the gel, avoiding air bubbles. The membrane was then covered with 2 pieces of Whatman filter paper soaked in 2X SSC, of the same size as the gel and with Saran wrap, which were big enough to extend to the sides of the glass tray. The region of Saran wrap overlying the gel was cut away using a scalpel and a wad of absorbent tissue placed above the gel and filter paper. A glass plate was placed covering the set and finally a weight of 500 g was added on top. After overnight transfer the nylon membrane was rinsed in 2X SSC, allowed to air dry for 1 hour and fixed by the alkali

transfer method. For this, 2 sheets of Whatman 3mm paper were placed in a plastic container and soaked in 0.4N NaOH. Dry membranes were placed DNA side up on the Whatman paper for 20 minutes and then briefly rinsed in 5X SSC to remove excess NaOH. Membranes were immediately used for hybridisation.

4. 2. 4. 4 DNA Hybridisation

Specific xylanase and arabinosidase probes designed by PCR as described in 4.2.2 were used to hydridise to restriction fragments of *S. nodorum* DNA on southern blots. The probes were radiolabelled with [α-^{32}P]dCTP using the Pharmacia Biotech "Oligolabelling kit". For preparation, 40 µl containing 50 -100 ng of DNA were mixed with 10 µl 5X reaction buffer, 3µl radiolabelled dCTP and 1µl Klenow, following the specifications of the manufacturer. Labelled probe was denatured by heating to 100°C for 5 minutes and snap cooling on ice for 2 minutes. Unincorporated nucleotides were removed by passing through a 2 ml Sephadex G-50 column.

Membranes were put inside hybridisation tubes (4 x 25 cm, HYBAID Ltda) and pre-hybridised by washing in excess with hybridisation solution [5.9% PEG-600 (Fluka), 6.65% SDS (BDH) dissolved in 250mM sodium-phosphate buffer pH 7.2 (Na_2HPO_4, Sigma), and prepared as shown in appendix 17]. Pre-hybridisation was carried out at 60°C in a rotisserie oven (Hybaid Ltd) for at least 1h, after which the prehydridisation solution was poured off and replaced with a small volume of fresh hybridisation solution. Labelled denatured probe was then added and hybridisation was allowed to proceed overnight at the same temperature. Membranes were washed twice for 30 min, with low stringency wash solution (1X-hybridisation solution), to remove unbound probe. These were followed by medium stringency wash solution (0.5X-hybridisation solution) and finally for high stringency wash solution (0.25X-hybridisation solution). All washes were carried out at 60 °C for 30 minutes. Medium and high stringency wash solutions were prepared by dilution of the hybridisation solution with distilled water. Low, medium and high stringency solutions contained 250mM, 125mM and 62.5mM sodium ions respectively, and are equivalent to 1.7X, 0.8X and 0.2X SSC respectively. Membranes were then removed, excess liquid blotted off, wrapped in saran wrap and placed in an autoradiography cassette (Genetic

Research Instrumentation Ltd) with intensifying screens. The membranes were covered with a sheet of X-ray film (Blue sensitive X-ray film, Genetic Research Instrumentation Ltd) and autoradiograph cassettes were stored at - 70 °C. Autoradiographic films were developed using an X-omat 2X processor.

4. 2. 5 cDNA Library Construction

Fungal RNA extraction, poly $(A)^+$ preparation and cDNA synthesis were made by Dr. L.V. Bindschedler. In short, an established biomass of mycelium of *S. nodorum* was grown on a medium containing cell wall for 16h, 27h and 40h. After centrifugation and decanting, about 6 to 8g of insoluble material (mycelium and cell walls) was fast frozen and ground. RNA was extracted with Tri-reagent solution (Gibco) according to the manufacturer's protocol. $PolyA^+$ RNA was purified with PolyAttract mRNA Isolation SystemIV (Promega, Madison, USA). Five micrograms of $PolyA^+$ RNA (2.5µg of the 16h-time point, 1.25µg each for the 27h and 40h time points) were used for the cDNA synthesis with the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Fifty nanograms of cDNAs was ligated into the Uni-ZAP XR Vector Kit (Stratagene). λZAPII recombinant DNA was packaged into ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). The primary library that contains $4x10^6$ pfu was amplified. About $4x10^4$ pfu were plated for first screening

4. 2. 6 Screening of the cDNA Library

For the first screening, the library was plated on eight 14-cm diameter NZY agar petri dishes, mixing host cells and Lambda phage particles, to give 40000 plaque-forming units (pfu) per plate.

Host cells were the XL1-Blue MRS' *E. coli* strain. A single colony was inoculated in 10 ml LB broth supplemented with 10mM $MgSO_4$, 2% (w/v) maltose and 12.5µg /ml tetracycline. Cultures were incubated on a shaking incubator at 37 °C until an OD_{600} of 0.5 to 1.0 was achieved (usually 6 hours). The bacteria were then pelleted by

centrifugation at 1000 rpm for 10 min and the supernatant decanted. Cells were suspended in 10 ml of filter sterile 10 mM ice cold $MgSO_4$ and stored at 4 °C.

Lambda phage particles were diluted in SM buffer to give the desired pfu/ml. Volumes of 300 µl of cell suspension and of 20 µl of diluted phage were mixed in 20 ml glass test tubes. Mixtures were incubated at 37 °C for 15 min, allowing adsorption of the phage to the cells. Immediately after incubation, 6 ml of molten top agarose at around 45°C was added to each tube and gently mixed before pouring on to a pre-warmed LB agar plate. The plates were allowed to set for at least 15 minutes and incubated at 37 °C for 12-16 hours. Plates for screening were stored at 4 °C for at least one hour. Nylon membranes (Hybond N+) were cut to fit the 14 cm diameter plates containing the plaques. Then, membranes were placed over the surface of the agar plates for 1 minute. Replicas were made for each plate, leaving the replica membrane over the surface of the agar plate for 2 min. Each membrane and replica was marked properly for orientation, transferred with a forceps and placed plaque side up for 7 minutes on a piece of Whatman 3MM paper soaked in denaturing solution. The membrane was then transferred to Whatman 3MM paper soaked in neutralising solution for 3 minutes. This step was repeated once, and the membrane was rinsed briefly in 2X SSC and allowed to air dry for one hour. DNA fixation was carried out by the alkali transfer method described in 4.2.4.3.

Four membranes were hybridised with the xylanase probe and 4 with the arabinosidase probe, as described in 4.2.4.4. After developing the films exposed to the membranes, the corresponding positive spots present in each membrane and its replica were marked and matched on the plates, using the orientation marks. The positive plaques were cut and transferred into a 1.5-ml microcentrifuge tube containing1 ml of SM buffer and two drops of HPLC grade chloroform, used as a preserver. The clones in SM buffer were diluted and titred in 9 cm diameter NYZ agar petri dishes. For this, 160 µl of host cells were mixed with 10 µl of diluted phages, to give 400 plaques per plate. Mixtures were incubated at 37 °C for 15 min, and 3 ml of molten top agarose at around 45°C was added to each tube and gently mixed before pouring on to a pre-warmed 9 cm LB agar plate. Plates were allowed to set, incubated overnight and cooled as described. One membrane and replica were prepared for each plate,

prepared, fixed and hybridised as described for the first round of screening. Positives on the each membrane and replica were marked and positive plaques cut from the agar and transferred to 1ml SM buffer. The whole process was repeated for a third round of screening by plating the appropriate dilution of Lambda phage to give 40 plaques per plate.

4. 2. 6. 1 *In Vivo* Excision, Plasmid DNA Preparation and Sequencing of Xylanase and Arabinosidase Clones

Single positive plaques from the third screening were transferred to 500 µl SM buffer and two drops of chloroform and kept overnight at 4 °C. Overnight liquid cultures of XL1-Blue MRF' and SOLR *E. coli* strains were made in LB supplemented with maltose (0.2%) and $MgSO_4$ (10 mM). Cultures were spun down at 1000 rpm for 10 min, and the cells suspended in 10mM $MgSO_4$ to give an OD_{600} of 1.0. Then the following mixture was set up in 15 ml sterile polypropylene tubes and incubate at 37 °C for 15 min: 200 µl XL1-Blue, 250µl of each phage stock (>10^5 pfu) and 1 µl provided Ex Assist (>10^6 pfu). After incubation, 3 ml of fresh LB were added to each tube and the cultures grown on an orbital shaker at 37 °C and 200 rpm for 3 h. The tubes were heated up to 65-70 °C for 20 min, then spun down at 1000 xg for 15 min. The supernatants containing the excised pBluescript phagemid as filamentous phage particles were transferred into new polypropylene tubes and stored at 4 °C. In order to plate each excised phagemid preparation the following two mixtures were prepared using an overnight culture of SOLR re-suspended on 10 mM $MgSO_4$ to give an OD_{600} of 1.0 : tube A (200 µl SOLR and 100 µl phage) and tube B (200 µl SOLR and 10 µl phage). The tubes were incubated at 37°C for 15 min and later plated on LB-ampicillin (100µg /ml). Plates were incubated at 37°C overnight. Single colonies were then isolated from each clone and again, plated on agar slants for conservation at 4 °C and also in liquid LB broth for DNA plasmid isolation.

Plasmid DNA was prepared from each selected single colony, by using a QIAprep Spin miniprep kit, and following manufacturer's instructions. All clones were sent to be sequenced with the universal primers T3 and T7 to get information of the 5' and 3' regions of the plasmids.

4. 3 Results

Using the databases to get information of xylanases and arabinosidases sequences from bacteria and fungi, DNA and RNA strategies were developed in order to find the genes that codify for these activities in *S. nodorum* and in doing so increasing the knowledge of the CWDE of this fungus at the molecular level.

4. 3. 1 Design of Probes and Screening of cDNA Library for Xylanase Clones

Based on the alignment of protein and nucleotide sequences of fungal xylanases shown in Fig 35 and Fig 36 respectively, three regions of high amino acid and nucleotide sequence conservation were identified and named: xylanase forward (XF), xylanase internal (XI) and xylanase reverse (XR).

XF-XI spanned a region of approximately 82 amino acids and 246 bp. XF-XR spanned a region 108 amino acids and 324 bp. Oligonucleotides were designed based on these regions:

XF: 5'- ATCAACTTCTCCGGCACCT – 3',
XI: 5'- CGCGAGCCCTGTCGAACGGA - 3' and
XR: 5'- CCTCTACCGCTACGACCTG - 3'.

These oligonucleotides were used in PCR reactions in order to amplify DNA fragments from genomic DNA of the four *S. nodorum* isolates BS 171, BS 471, LAW and LN 97. The conditions for PCR that were finally standardised were as follows:

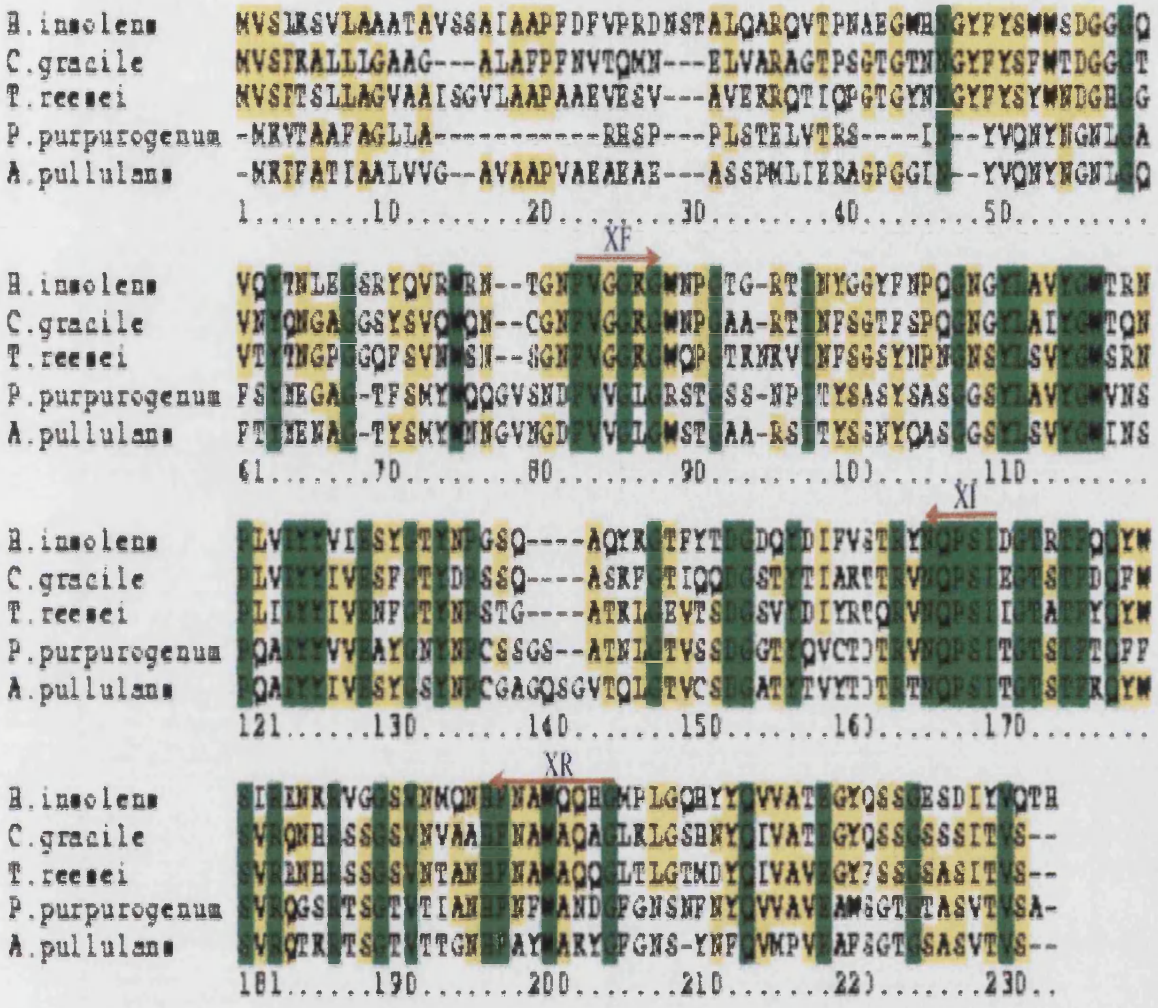


Figure 35 : Multiple protein sequence alignment for the designing of xylanase probe. Xylanses from: *Humicola insolens*, *Chaetomium gracile*, *Trichoderma reesei*, *Penicillium purpurogenum* and *Aureobasidium pullulans* are aligned. Positions of the amino acids in the primary sequences are shown in the numbers below the blocks. Amino acid residues that exhibit identity in all five proteins are coloured in green, residues with 60 to 80% similarity are coloured in yellow. Red arrows indicate the position of the designed primers in the amino acid sequence: XF, xylanase forward; XI, xylanase internal and XR xylanase reverse.

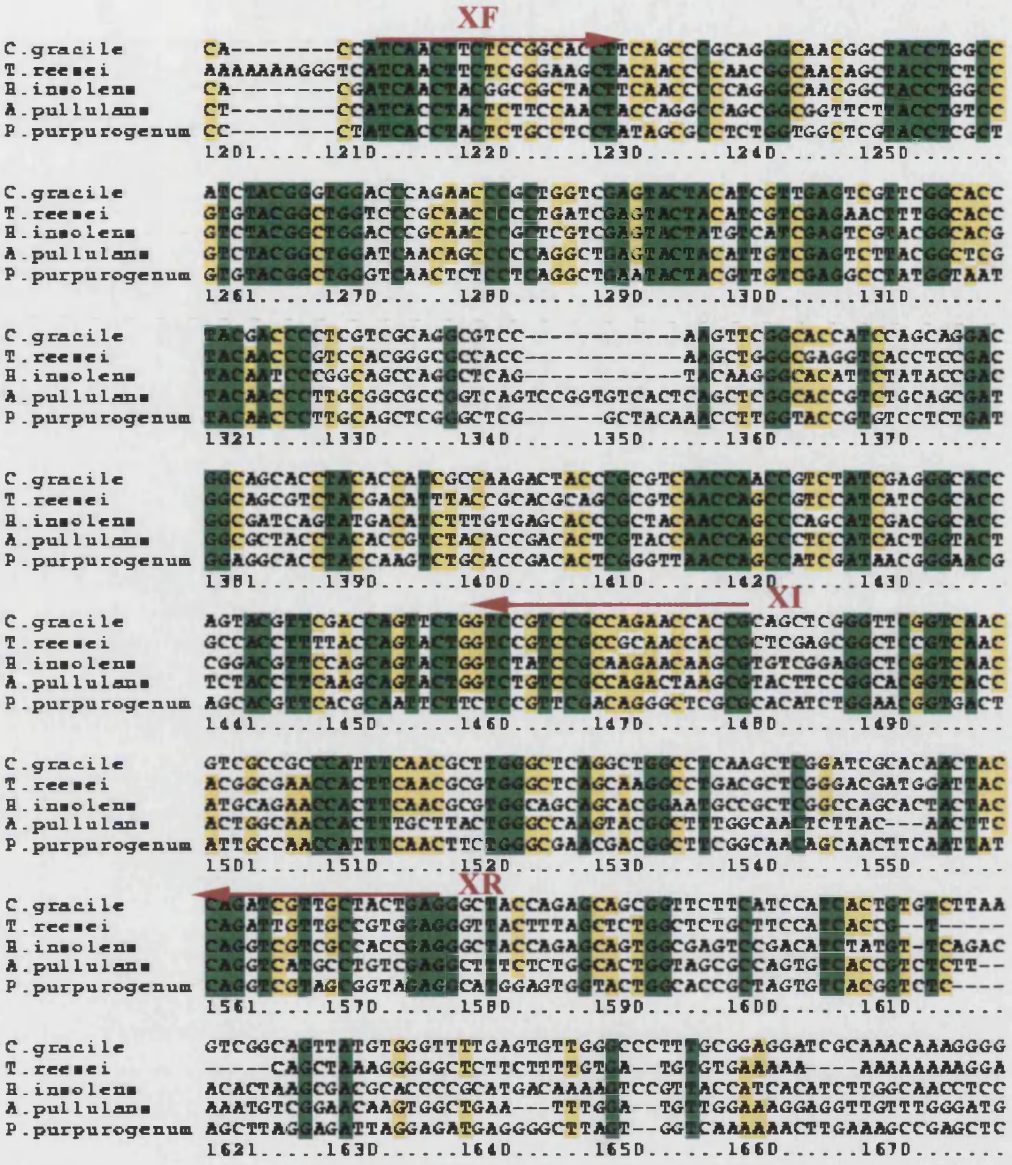


Figure 36 : Multiple DNA sequence alignment for the designing of xylanase probe. Xylanse genes from: *Humicola insolens*, *Chaetomium gracile*, *Trichoderma reesei*, *Penicillium purpurogenum* and *Aureobasidium pullulans* are aligned. Positions of the nucleotides in the primary sequences are shown in the numbers below the blocks. Nucleotide residues that exhibit identity in all five DNA sequences are coloured in green, residues with 60 to 80% similarity are coloured in yellow. DNA sequences corresponding to the primers designed in the amino acid sequence alignment are shown as red arrows: XF, xylanase forward; XI, xylanase internal and XR, xylanase reverse.

10 x Buffer	5 µl
Each primer	1µM
Template (DNA)	10 ng
MgCl	1.2 mM
dNTP	0.2 mM each
Taq polymerase	0.5 U
MilliQ water	To 50 µl

Melting	96 °C	5 min
Denaturing	96 °C	1 min
Annealing	57 °C	1 min
Extension	72 °C	1 min
40 Times cycle	Denaturing-extension	1 min 15 sec
End	4 °C	1 h

With these conditions, products of expected size of around 400 and 200 bp were obtained as is shown in Fig 37. These fragments were recovered from the gels (section 4.2.4.1) and were sent for sequencing using the XF oligo as the sequencing primer. The nucleotide and deduced amino acid sequences were submitted to a BLAST search as indicated in 4.2.2. It was confirmed that the amplified DNAs were indeed internal fragments of xylanase genes.

The PCR amplified products hybridised to a small number of restriction fragments under high stringency Southern blot suggesting a family of xylanase genes and related sequences in the *S. nodorum* genome as can be seen in Fig 38a and 38c.

A *S. nodorum* cDNA library prepared from mycelia grown for 16 to 40 h in a medium containing wheat cell wall as the sole carbon source was screened. The PCR product spanning the region XF–XR was used as a probe, under hybridisation stringencies as indicated in 4. 2. 4. 4.

For first round screening 1.6 x 10^4 recombinant clones were examined. Following three successive rounds of hybridisation, five positive clones designated 1X to 5X were obtained. Plasmids were excised *in vivo* as indicated in 4.2.7.1 The plasmids were purified (Fig 39a) and sent to be sequenced in both 5' and 3' ends using the universal

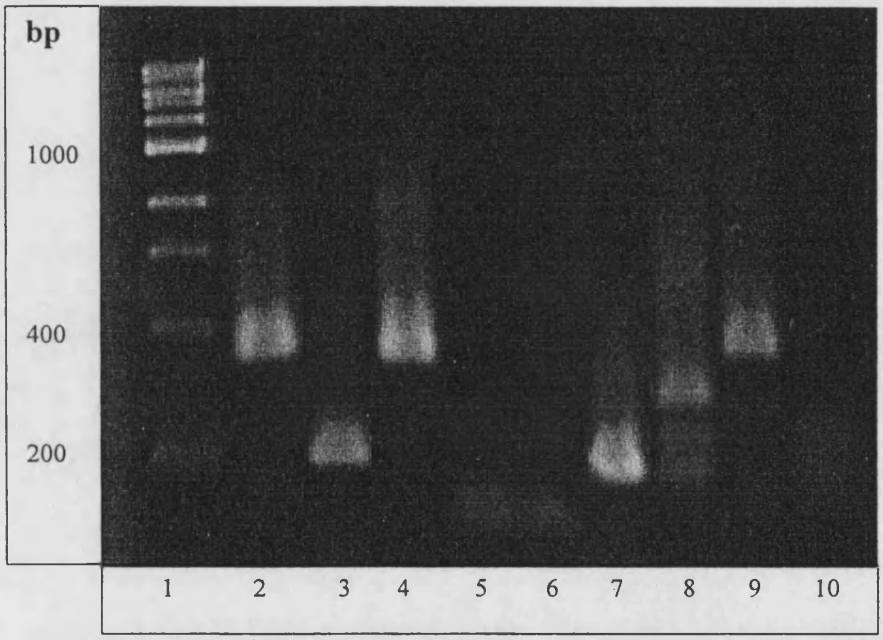


Figure 37 : PCR products of *S. nodorum* genomic DNA from different isolates at 57 °C annealing temperature, using the primer combinations XF-XR (**Xfr**) and XF-XI (**Xfi**) designed to amplify regions in the xylanase gene of around 324 bp and 246 bp respectively. Lane 1, Hyper ladder I (Bioline) DNA size marker; lanes 2 and 3 **Xfr** and **Xfi** for BS 471; lanes 4 and 7 **Xfr** and **Xfi** for BS171; lanes 8 and 9 **Xfi** and **Xfr** for LAW; lanes 5, 6, and 10 are PCR reaction controls.

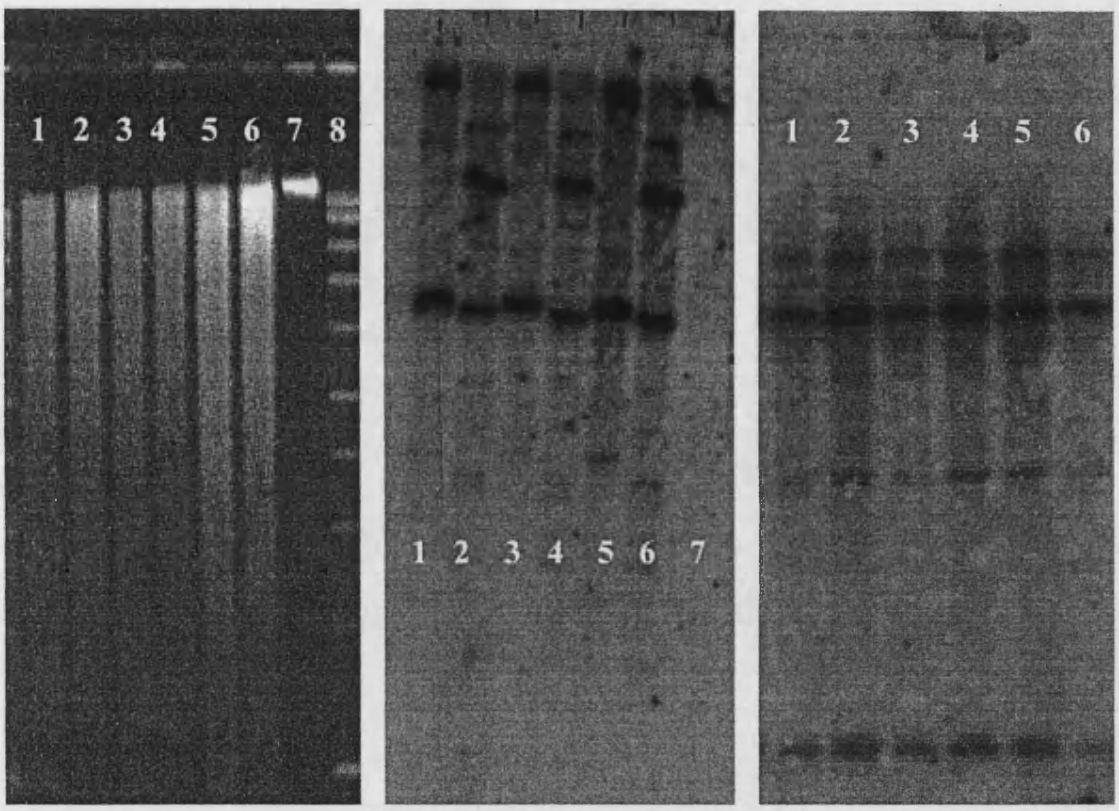


Figure 38 : Southern blot analysis of *S. nodorum* genomic DNA (20 μg per lane), after digestion with different restriction endonucleases and electrophoresis at 1.5 v cm^{-1} on 0.8% TAE gel. DNA of LN 97 (lanes 1 and 2), LAW (lanes 3 and 4) and BS 171 (lanes 5 and 6) were digested with *Hind* III (lanes 1, 3 and 5) and *Eco* RV (lanes 2, 4 and 6) while lanes 7 and 8 were undigested DNA and DNA size marker respectively **(a)**, according to standard procedures. The blot was hybridised with PCR purified arabinosidase **(b)** and xylanase **(c)** probes.

(a) Plasmids containing arabinosidase and xylanase genes

```
5 1x31                          +------------------->
4 1xt7                         <--------------------+
3 1x.t3          +------------------->
2 1x71           <-------------------+
C CONSENSUS      <-----------------------------------+
                 |----------|----------|----------|---------|---------|
                0          400        800       1200       1600


5 5x.31                        +-------------->
4 5x.t7                        <-----------------+
3 5x.71          <---------------------+
2 5x.t3          +------------------->
C CONSENSUS      +---------------------------------->
                 |----------|----------|----------|---------|---------|
                 0          400        800       1200       1600
```

(b) Sequencing strategy for xylanase clones 1x and 5x

Figure 39 : (a) Purified plasmids containing xylanase and arabinosidase genes for sequence: clones 5x, 4x , 3x and 1x are in lanes 2 to 5 respectively; clones 6a, 7a, 8a, 9A, 10a and 11a are in lanes 8 to 13 respectively. **(b)** The GCG program GELASSEMBLE output showing sequencing strategy for xylanase clones 1x and 5x. Universal T3 and T7/pUC primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotides primers were designed based on sequence data obtained, using the "Primer designer" software programme.

T3 primers present in the plasmid pBluescrip KS (Stratagene) in which phagemids were excised.

Sequences at both ends were compared for similarities using the Clustal W program (biology work bench at http://workbench.sdsc.edu). Three groups of identical sequences were found among the clones as follow: 1x alone; 2x and 3x; and finally 4x and 5x. One clone for each group was chosen for further sequencing. Unfortunately, it was not possible to obtain full sequence from clones 2x or 3x. Sequencing of clones 1x and 5x was completed by both ends as shown in Fig 39b.

4. 3. 2 Design of Probes and Screening of cDNA Library for Arabinosidase Clones

Based on the alignment of protein and nucleotide sequences of fungal and bacterial xylanases shown in Fig 40 and Fig 41, three regions of high amino acid and nucleotide sequence conservation were identified and named: arabinosidase forward (AF), arabinosidase internal (AI) and arabinosidase reverse (AR).

AF-AI spanned a region of approximately 124 amino acids and 372 bp and AF–AR spanned a region of 200 amino acids and 600 bp. Oligonucleotides were designed based on these regions:

AF: 5'- ATCTGG(G,A)TTCTGGCCTA(G,C)–3'
AI: 5'-TGGCCGTGAAGGAGCGGAA-3'
AR: 5'-A(G,A)GGTA(G,A)GG(G,A)(T,C)GCCCGG -3'.

These oligonucleotides were used in PCR reactions in order to amplify DNA fragments from genomic DNA of the four *S. nodorum* isolates BS 171, BS 471, LAW and LN 97. The conditions for PCR that were finally standardised are as follows:

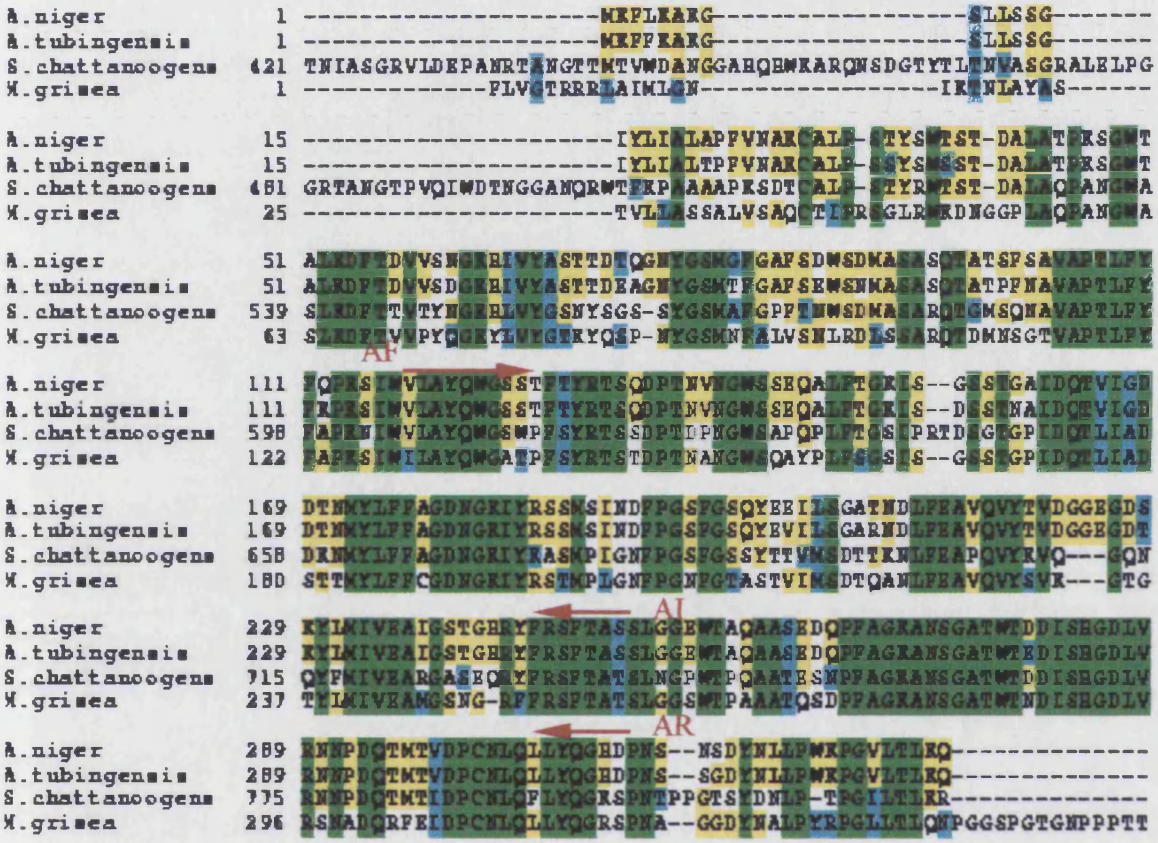


Figure 40 : Multiple protein sequence alignment for the designing of arabinosidase probe. Arabinosidases from: *Aspergillus niger*, *Aspergillus tubingensis*, *Streptomyces chattanoogens*, and *Magnaporthe grisea* are aligned. Positions of the amino acids in the primary sequences are shown in the numbers at the left of the blocks. Amino acid residues that exhibit identity in all five proteins are coloured in green, residues with 60 to 80% similarity are coloured in yellow. Red arrows indicate the position of the designed primers in the amino acid sequence: AF, arabinosidase forward; AI, arabinosidase internal and AR, arabinosidase reverse.

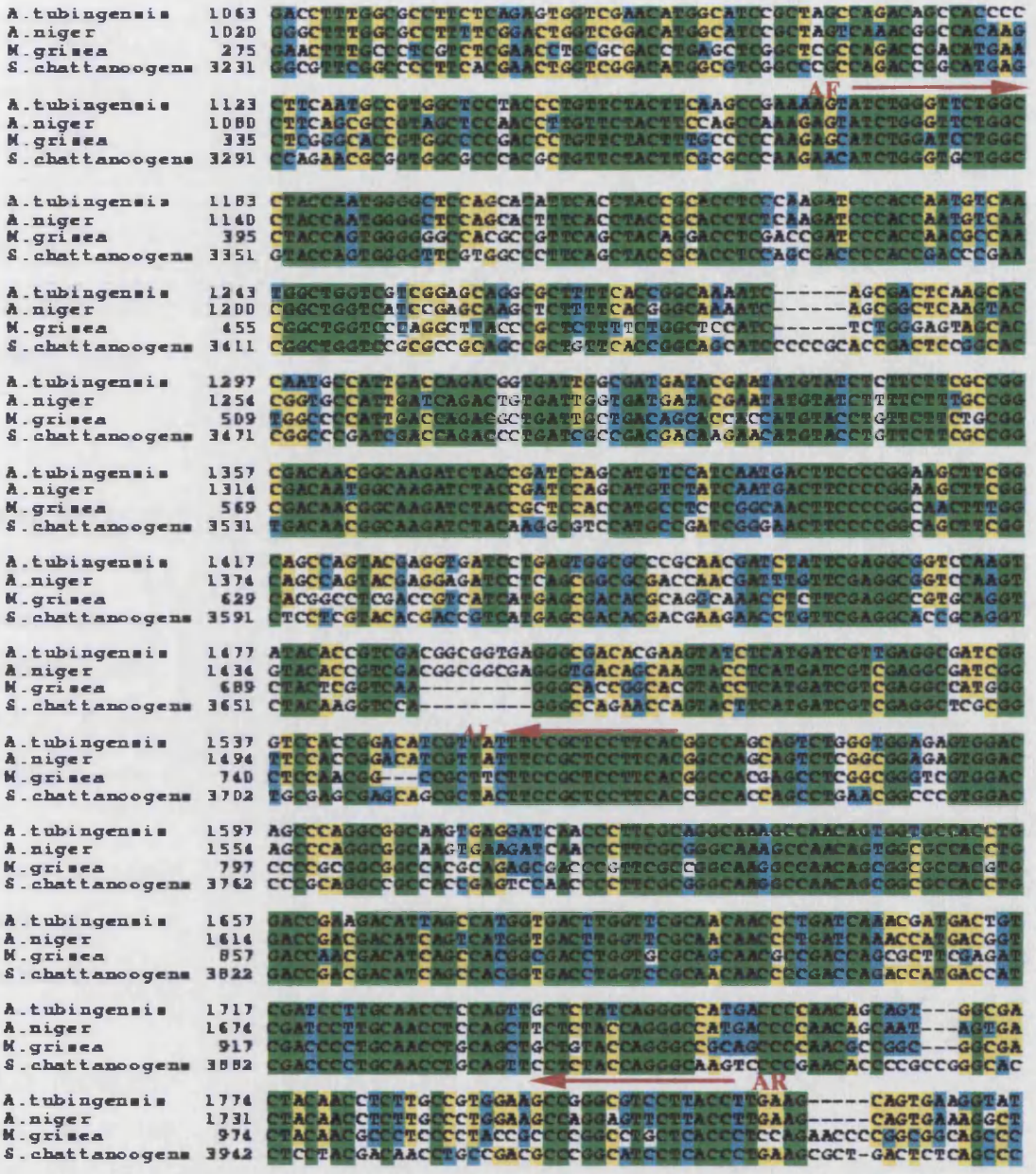


Figure 41 : Multiple DNA sequence alignment for the designing of arabinosidase probe. Arabinosidases genes from: *Aspergillus niger*, *Aspergillus tubingensis*, *Streptomyces chattanoogens*, and *Magnaporthe grisea* are aligned. Positions of the nucleotides in the primary sequences are shown in the numbers at the left of the blocks. Nucleotide residues that exhibit identity in all five DNA sequences are coloured in green, residues with 60 to 80% similarity are coloured in yellow. The DNA sequences corresponding to the primers designed in the amino acid sequence alignment are shown as red arrows: AF, arabinosidase forward; AI, arabinosidase internal and AR, arabinosidase reverse.

10 x Buffer	5µl
Each primer	1µM
Template(DNA)	10 ng
MgCl	1.5 mM
dNTP	0.2 mM each
Taq polymerase	0.5 U
MilliQ water	To 50 µl

Melting	96 °C	5 min
Denaturing	96 °C	1 min
Annealing	46 °C	1 min
Extension	68°C	1 min
40 Times cycle	Denaturing-extension	1 min 15 sec
End	4 °C	1 h

With these conditions, products of expected size around 600 and 400 were obtained as shown in Fig 42. These fragments were recovered from the gels (section 4.2.4.1) and were sent for sequencing using the AF oligo as the sequencing primer. The nucleotide and deduced amino acid sequences were submitted to a BLAST search as indicated in 4.2.2. It was confirmed that the amplified DNAs were indeed internal fragments of arabinosidase genes.

The PCR amplified products hybridised to a small number of restriction fragments under high stringency Southern blot suggesting a family of arabinosidase genes and related sequences in the *S. nodorum* genome as can be seen in Fig 38b.

A S. *nodorum* cDNA library prepared from mycelia grown in media containing wheat cell wall as the sole carbon source was screened. The PCR product spanning the region AF–AR was used as a probe, under hybridisation stringencies as indicated in 4.2.4.4. For first round screening 1.6 x 10^4 recombinant clones were examined. Following three successive rounds of hybridisation, six positive clones designated 6A–11A were obtained. Plasmids were excised *in vivo* as indicated in 4.2.7.1 The plasmids were purified (Fig 39) and sent to be sequenced in both 5' and 3' ends using the universal T7 and T3 primers present in the plasmid pBluescrip KS (Stratagene) in which phagemids were excised.

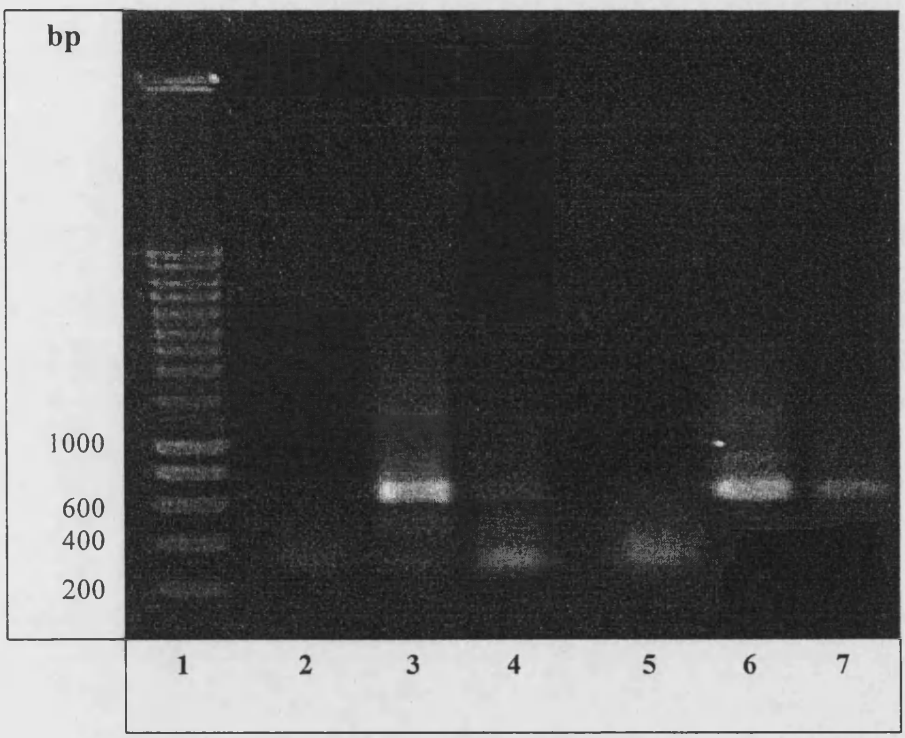


Figure 42 : PCR products of *S. nodorum* genomic DNA from different isolates at 46°C annealing temperature, using the primer combinations AF-AR (Afr) and AF-AI (**Afi**) designed to amplify regions in the arabinosidase gene of around 600 bp and 372 bp respectively. Lane 1, Hyper ladder I (Bioline) DNA size marker; lanes 2 and 7 **Afi** and Afr for BS 471; lanes 3 and 4 Afr and **Afi** for BS171; lanes 5 and 6 **Afi** and Afr for LAW.

Sequences at both ends were compared for similarities using the Clustal W program (biology work bench at http://workbench.sdsc.edu). Three groups of identical sequences were established among the clones as fallows: 6A-7A, 8A-10A, and 9A-11A. One clone for each group was chosen for further work. Sequencing of clones 6a, 8a and 9a was completed by both ends as shown in Fig 43.

4. 3. 3 Analysis of DNA and deduced amino acid sequences for xylanase clones

The sequence and deduced translation of a *S. nodorum* xylanase 1 is shown in Fig 44. The clone was designated **SncXYI** (*Stagonospora nodorum* cDNA encoding xylanase I) and is to be submitted to the Genbank database. The clone is 925 bp in size and encodes a predicted protein of 247 amino acids.

The nucleotide sequence contains a short 5' UTR with the core of a capping site motif CATTC (Legerton and Yanofsky, 1985) and the start codon located in the sequence context **CACAATGG**, similar to the Kozak consensus **CCGCCATGG** identified as optimal for eukaryotic translation initiation (Hinichs, 1995). The 3' UTR contains an AATGAA similar to the polyadenylation signal (consensus AATAAA) located 11 bp upstream of the poly (A) addition site.

Computer analysis of the deduced protein indicates a molecular weight of 27,490 kDa and a predicted isoelectric point of 9.2. Analysis with PSORT and related programmes indicate the presence of the following motifs: Glycosyl hydrolase family 11 active site signatures 1 and 2, hydrolase xylan glycosidase degradation precursor and endo-1 4-β-xylanase signal. The sequence shows high probability of a signal peptide of 19 residues, being the most likely cleavage site between position 19 and 20. It also contains an N-glycosylation site in residues 32-35 (NATA) and appears to be an extracellular protein (including cell wall, P= 0.56) and /or to be located in the endoplasmic reticulum (P= 0.22).

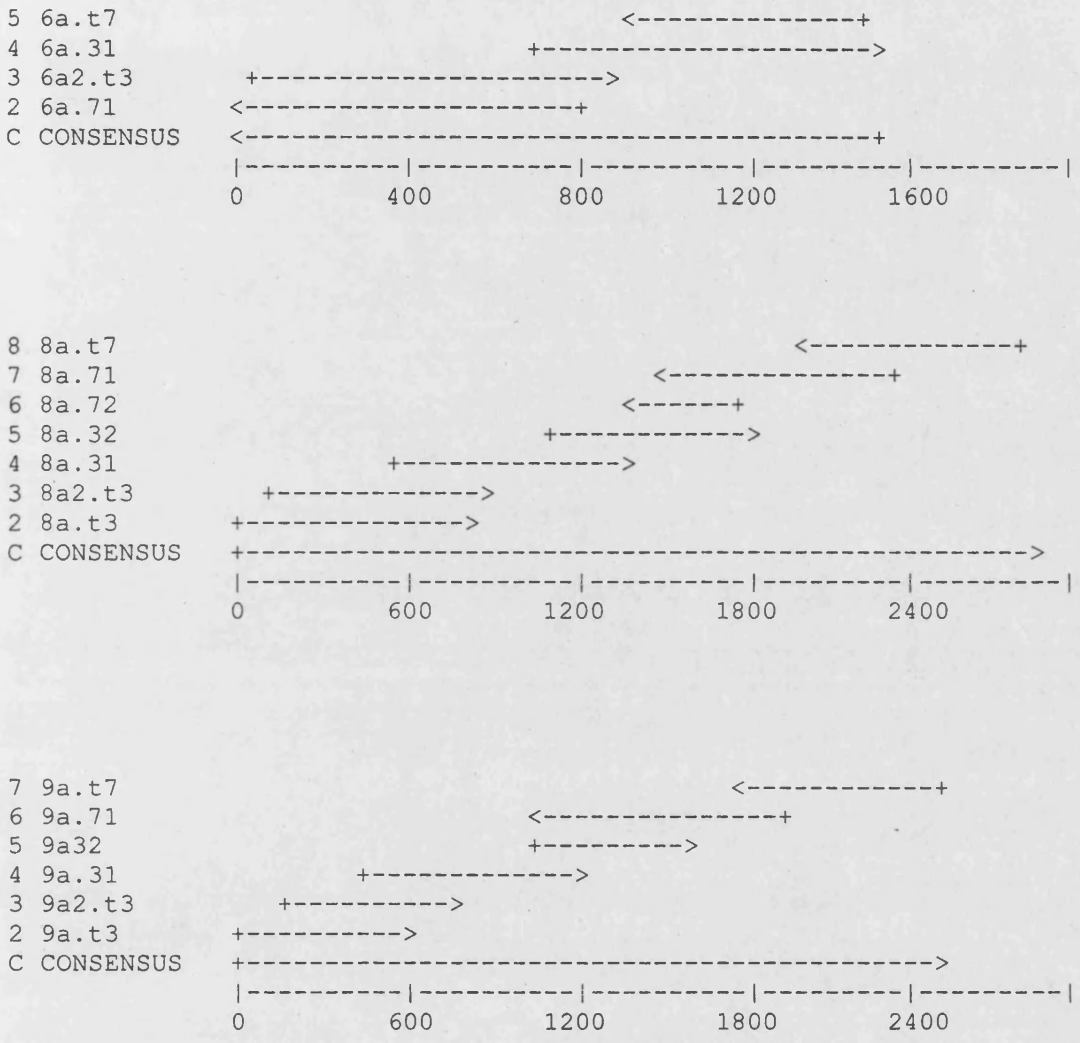


Figure 43 : The GCG program GELASSEMBLE output showing sequencing strategy for arabinosidase clones 6a, 8a and 9a. Universal T3 and T7/pUC primers were used for initial forward and reverse sequencing reactions. For subsequent internal sequencing reactions oligonucleotides primers were designed based on sequence data obtained, using the "Primer designer" software programme.

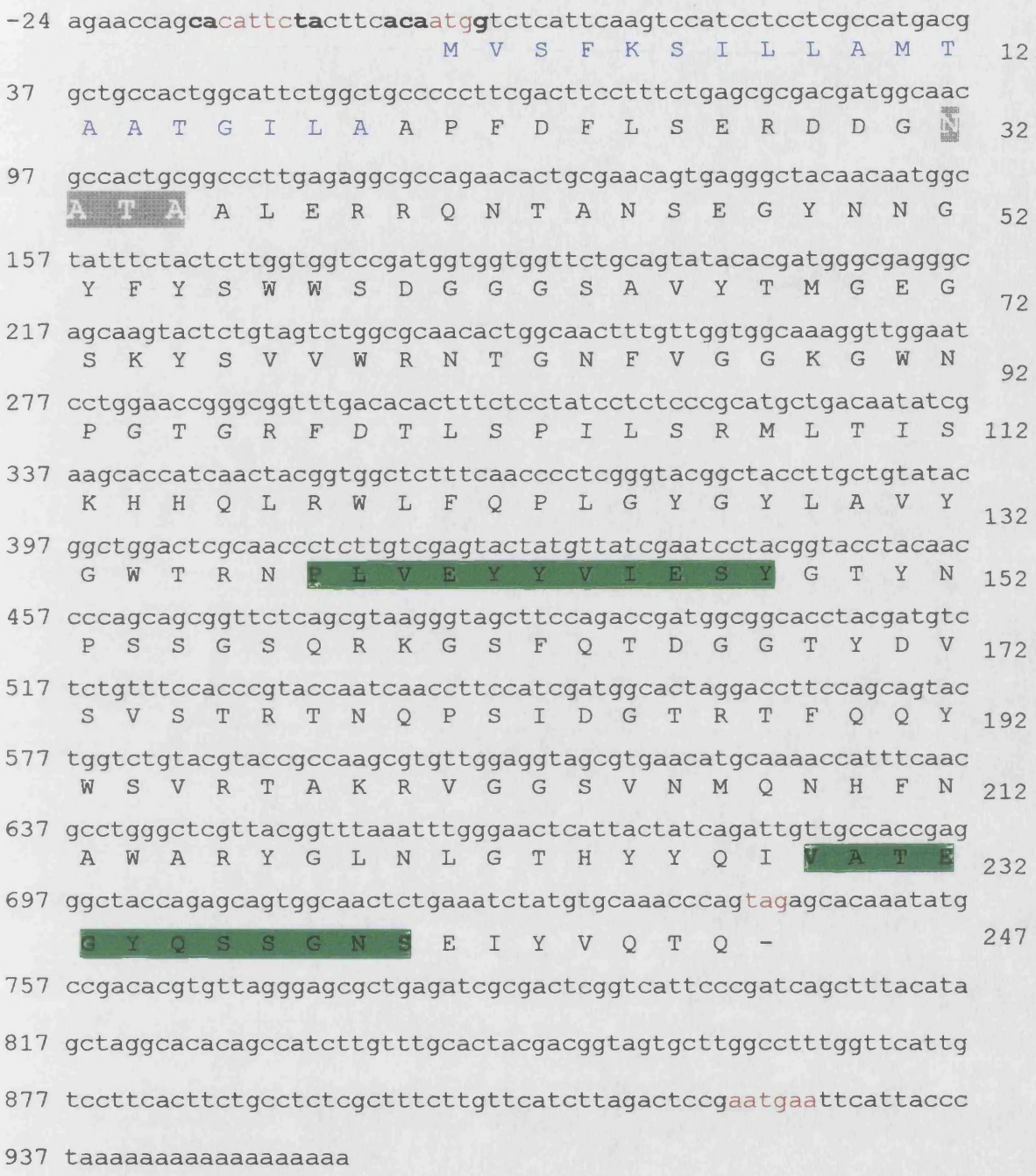


Figure 44 : Nucleotide sequence and deduced translation of *S. nodorum* xylanase I (cDNA clone **SncXYI**). Numbers to the left indicate nucleotide position relative to the A nucleotide in the initiation codon (ATG). Those to the right represent the amino acid position. Nucleotide sequence features: a core of a capping site motif, the start ATG codon within a Kozak consensus, the predicted stop codon and the putative polyadenylation signal are shown in red in respective order 5'-3'. Within the deduced polypeptide sequence, a putative signal peptide is shown in blue; the catalytic sites 1 and 2 (family 11) are highlighted in green, and a glycosylation site is highlighted in grey.

The sequence and deduced translation of a *S. nodorum* xylanase 5 is shown in Fig 45. The clone was designated **SncXYII** (*Stagonospora nodorum* cDNA encoding xylanase II) and is to be submitted to the Genbank database. The clone is 809 bp in size and encodes a predicted protein of 229 amino acids.

The nucleotide sequence does not contain the start codon **ATG**, probably due to the loss of the 5' end of the mRNA at the time of the library construction. The 3' UTR contains an AATACGAA similar to the polyadenylation signal (consensus AATAAA) located 20 bp upstream of the poly (A) addition site.

Computer analysis of the deduced protein indicates a molecular weight of 24,660 kDa and a predicted isoelectric point of 9.0. Analysis with PSORT and related programmes indicates the presence of the following motifs: Glycosyl hydrolase Family 11 active site signature 1 and active site signature 2, hydrolase xylan glycosidase degradation precursor and endo-1 4-β-xylanase signal.

The sequence shows high probability of a signal peptide of 18 residues, being the most likely cleavage site between positions 18 and 19. It also contains an N-glycosylation site in residues 95-98 (NVTY). The protein appears to be extracellular (including cell wall, P= 0.68) and/or vacuolar (P= 0.22).

4. 3. 4 Analysis of DNA and deduced amino acid sequences for arabinosidase clones

The sequence and deduced translation of the *S. nodorum* arabinosidase clone 6 is shown in Fig 46. The clone was designated **SncARI** (*Stagonospora nodorum* cDNA encoding arabinosidase I) and is to be submitted to the Genbank database. The clone is 1058 bp in size and encodes a predicted protein of 324 amino acids.

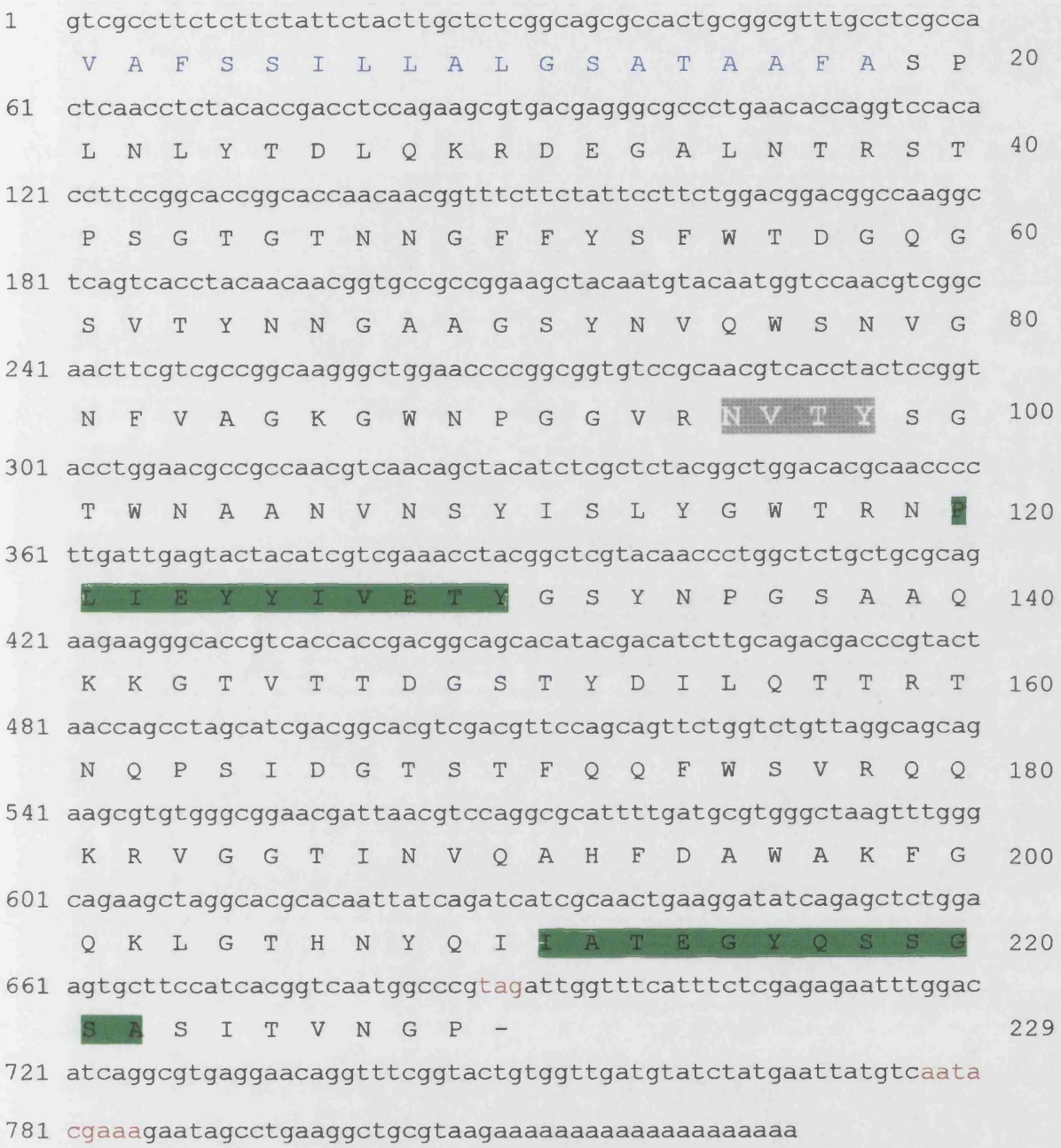


Figure 45 : Nucleotide sequence and deduced translation of *S. nodorum* xylanase II (cDNA clone **SncXYII**). Numbers to the left indicate nucleotide position relative to the G nucleotide in the codon of the first amino acid (GTC). Those to the right represent the amino acid position. Nucleotide sequence features: predicted stop codon and the putative polyadenylation signal are shown in red in respective order 5'-3'. Within the deduced polypeptide sequence, a putative signal peptide is shown in blue and the two catalytic sites 1 and 2 (family 11) are highlighted in green. A glycosylation site (NVTY) is highlighted in grey.

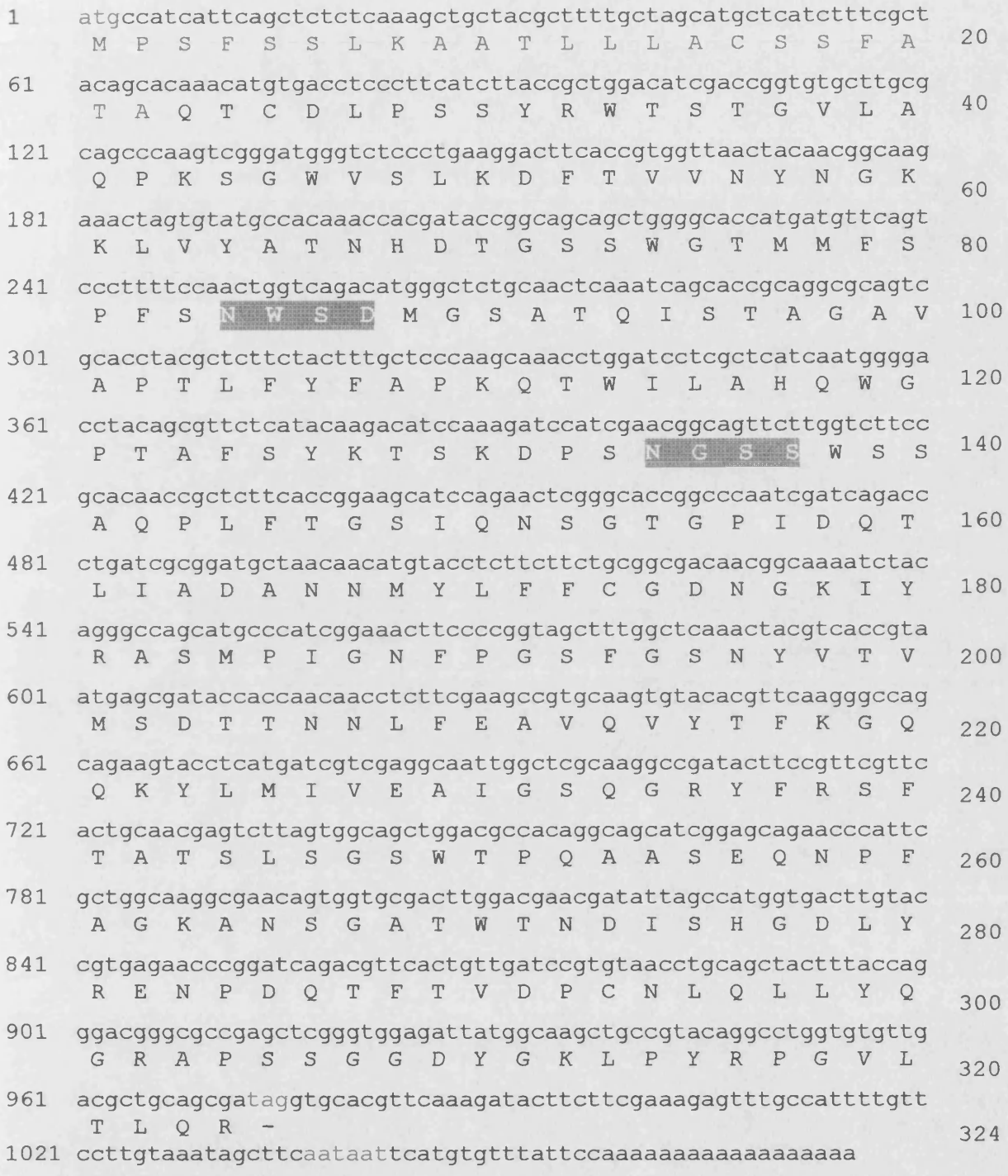


Figure 46 : Nucleotide sequence and deduced translation of *S. nodorum* arabinosidase I (cDNA clone **SncARI**). Numbers to the left indicate nucleotide position relative to the A nucleotide in the initiation codon (ATG). Those to the right represent the amino acid position. Nucleotide sequence features: the start ATG codon, the predicted stop codon and the putative polyadenylation signal are shown in red in respective order 5'-3'. The deduced polypeptide sequence has a putative signal peptide of 22 residues in blue, and 2 glycosylation sites, which are highlighted in gray.

The nucleotide sequence begins exactly with the start codon **ATG**. This may be due to the loss of the 5' UTR during the library construction. The 3' UTR contains an AATAAT similar to the polyadenylation signal (consensus AATAAA) located 15 bp upstream of the poly (A) addition site.

Computer analysis of the deduced protein indicates a molecular weight of 35, 173 kDa and a predicted isoelectric point of 8.5. Analysis with PSORT and related programmes did not show any special motifs. Nevertheless the sequence seems to contain a signal peptide of 22 residues, being the most likely cleavage site between position 22 and 23. The sequence also shows two N-glycosylation sites in residues 84-87 (NWSD) and in residues 134-137 (NGSS). The protein appears to be extracellular (including cell wall, P= 0.56) and/ or cytoplasmic (P= 0.22).

The sequence and deduced translation of the *S. nodorum* arabinosidase clone 8 is shown in Fig 47. The clone was designated **SncARII** (*Stagonospora nodorum* cDNA encoding arabinosidase II) and is to be submitted to the Genbank database. The clone is 1192 bp in size and encodes a predicted protein of 323 amino acids.

The nucleotide sequence contains a short 5' UTR with the start codon located in the sequence context **CGATGG**, similar to the Kozak consensus **GCAATGG** identified in *N. crassa* as start for translation initiation. The 3' UTR contains an exact polyadenylation signal (consensus AATAAA) located 16 bp upstream of the poly (A) addition site.

Computer analysis of the deduced protein indicates a molecular weight of 34, 865 kDa and a predicted isoelectric point of 9.1. The sequence shows high probability of a signal peptide of 21 residues, being the most likely cleavage site between positions 21 and 22. The protein appears to be extracellular (including cell wall, P= 0.423) and/or cytoplasmic (P= 0.23).

The sequence and deduced translation of the *S. nodorum* arabinosidase clone 9 is shown in Fig 48. The clone was designated **SncARIII** (*Stagonospora nodorum* cDNA encoding

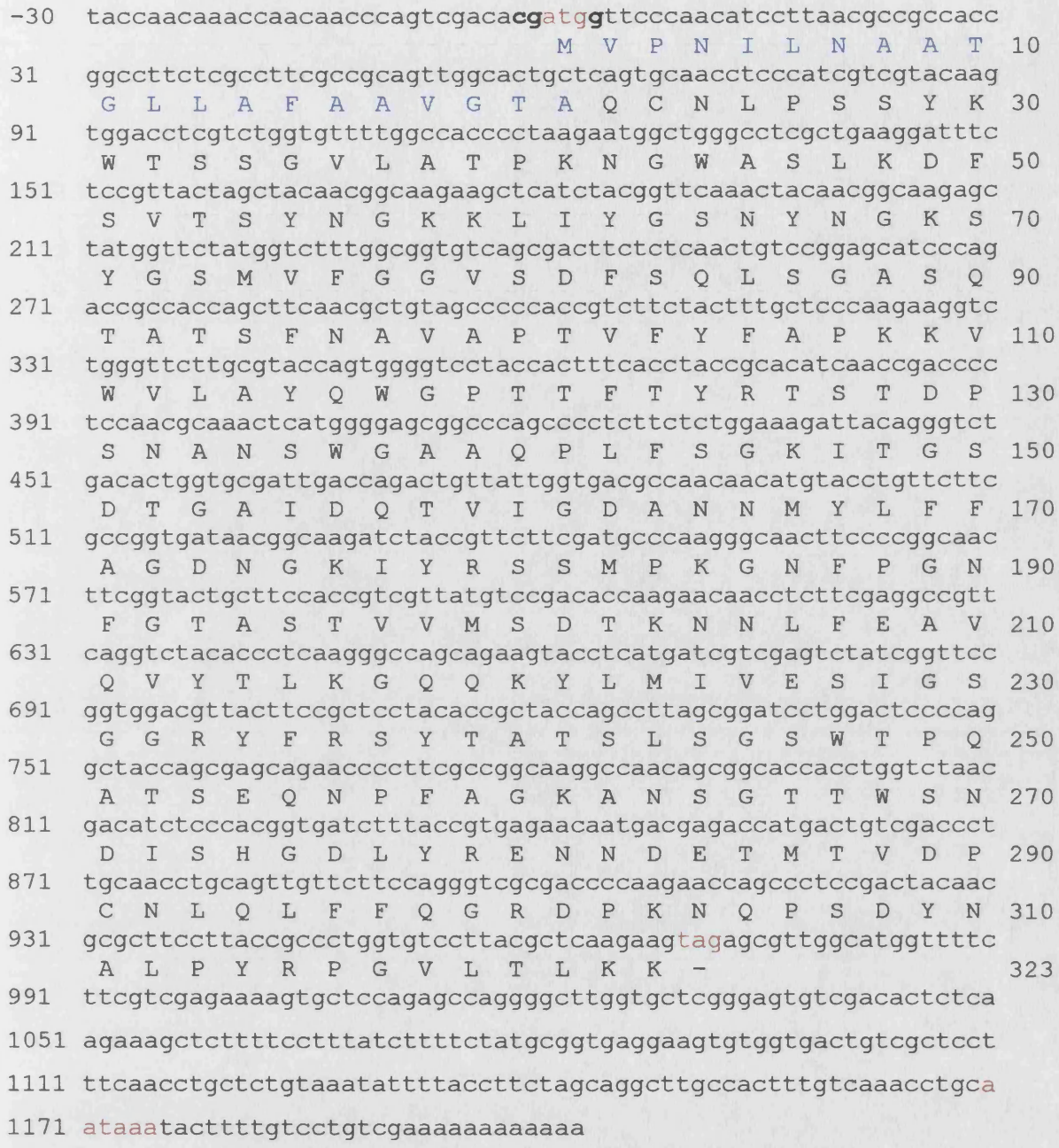


Figure 47 : Nucleotide sequence and deduced translation of *S. nodorum* arabinosidase II (cDNA clone **SncARII**). Numbers to the left indicate nucleotide position relative to the A nucleotide in the initiation codon (ATG). Those to the right represent the amino acid position. Nucleotide sequence features: the start ATG codon, the predicted stop codon and the putative polyadenylation signal are shown in red in respective order 5'-3'. The deduced polypeptide sequence has a putative signal peptide of 21 residues in blue.

```
522  ggaaagattacagggtctgacactggtgcgattgaccagactgttattggtgacgccaac
       G   K   I   T   G   S   D   T   G   A   I   D   Q   T   V   I   G   D   A   N  194
582  aacatgtacctgttcttcgccggtgataacggcaagatctaccgttcttcgatgcccaag
       N   M   Y   L   F   F   A   G   D   N   G   K   I   Y   R   S   S   M   P   K  214
642  ggcaacttccccggcaacttcggtactgcttccaccgtcgttatgtccgacaccaagaac
       G   N   F   P   G   N   F   G   T   A   S   T   V   V   M   S   D   T   K   N  234
702  aacctcttcgaggccgttcaggtctacaccctcaagggccagcagaagtacctcatgatc
       N   L   F   E   A   V   Q   V   Y   T   L   K   G   Q   Q   K   Y   L   M   I  254
762  gtcgagtctatcggttccggtggacgttacttccgctcctacaccgctaccagccttagc
       V   E   S   I   G   S   G   G   R   Y   F   R   S   Y   T   A   T   S   L   S  274
822  ggatcctggactccccaggctaccagcgagcagaacccttcgccggcaaggccaacagc
       G   S   W   T   P   Q   A   T   S   E   Q   N   P   F   A   G   K   A   N   S  294
882  ggcaccacctggtttaacgacatttcccacggtgatctttaccgtgagaacaatgacgag
       G   T   T   W   F   N   D   I   S   H   G   D   L   Y   R   E   N   N   D   E  314
942  accatgactgtcgacccttgcaacctgcagttgttcttccagggtcgcgaccccaagaac
       T   M   T   V   D   P   C   N   L   Q   L   F   F   Q   G   R   D   P   K   N  334
1002 cagccctccgactacaacgcgcttccttaccgccctggtgtccttacgctcaagaagaag
       Q   P   S   D   Y   N   A   L   P   Y   R   P   G   V   L   T   L   K   K   K  354
1062 agcgttggcatgtttttcttcgtcgagaaaagtgctccagagccaggggcttggtgctcg
       S   V   G   M   F   F   F   V   E   K   S   A   P   E   P   G   A   W   C   S  374
1122 ggagtgtcgacactctcaagaaagctcttttcctttatcttttctatgcggtgaggaagt
       G   V   S   T   L   S   R   K   L   F   S   F   I   F   S   M   R   -          391
1182 gtggtgactgtcgctcctttcaacctgctctgtaaatattttaccttctagcaggcttgc
1218 cactttgtcaaacctgcaataaatacttttgtcctgaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 48 : Nucleotide sequence and deduced translation of *S. nodorum* arabinosidase III (cDNA clone **SncARIII**). Numbers to the left indicate nucleotide position relative to the A nucleotide in the initiation codon (ATG). Those to the right represent the amino acid position. Nucleotide sequence features: capping site motif **CCATTC**, a transcription initiation motif **CAAT**, the Shine Delgarno sequence **GGAG**, the start codon located in the Kozak consensus sequence **GCAATGGC**, the polyadenylation signal **AATAAA**, the predicted stop codon and the putative polyadenylation signal **AATAAA** are shown in red, in respective order 5'-3'. The deduced polypeptide sequence has a putative signal peptide of 48 residues in blue.

```
-978 aatgtggtaatttcttgaaacaagctcagcatcaacctcggcatcgtcaggacgctaagt
-918 gcgtgcgcgggtttatgccttatacgtacgatgtttcctggggcatattacgatcaaagc
-858 gacctgtttgcgaatgcgctcggcgtctagtgtttcgaaggttgatcaaccaatggggca
-798 tgttcacagctgccgtggcaaaggttagactgaagacgtggatccacatggtatgaaacg
-738 tcgttcattaaaggacgggaggaacaatgtccaattcgaacaaccccaccagccacgcca
-678 tgcgctagtctccgacggtagctaaagaggcgagagtttctgggaaattcctgacaaaca
-618 cgtgtggatgcagcaatctaagagtatgcaccccagtcggcactaatcttccgacgtggc
-558 acagcaaccatatcctgcaccaaattgaaacgatcggggctccccatttgcctgaacatt
-498 cgtctggtcaaatctggggataattttcccccagtcatcgatgaccatgatgctaagctc
-438 ccttgttaaggctccgccgcttaaacaagcttcgcctctaagcttatggcgtccgtcact
-378 gtctctgagggaacccgaaacgagggagacttgccggcactagaggttcagcaaggcgtt
-318 taggaagtgcgatcaacttcctgttgctgcagaagcaccggctaatgctatacgatgggc
-258 gctgttgccattttgctcatggctgcatactcaagatcagattctcctggatgtccacga
-198 ctcacaaggtttccccgcgtgtcggagaaatccattccttccggtaatctcagtgttgcg
-138 ttgccggtggttcgagcagccttgtgtgaccccggtctcaatctcgaagcccaggcaagt
-78  ccagtgggcccaccaccttgtctacttggtactgcaatactagggtcgtagaccgaagac
-18  gggagcacacaataagcaatgctcttcacctgcggaacccacacccaaccacaacaggca
                    M  L  F  T  C  G  T  H  T  Q  P  Q  Q  A  14
42  ttcactcattaccaacaaaccaacaacccagtcgacacgatggttcccaacatccttaac
     F  T  H  Y  Q  Q  T  N  N  P  V  D  T  M  V  P  N  I  L  N  34
102 gccgccaccggccttctcgccttcgccgcaattggcactgctcagtgcaacctcccatcg
     A  A  T  G  L  L  A  F  A  A  I  G  T  A  Q  C  N  L  P  S  54
162 tcgtacaagtggacctcgtccggtgttttggcccaccccaaagaatgggctgggccttcg
     S  Y  K  W  T  S  S  G  V  L  A  H  P  K  E  W  A  G  P  S  74
222 ctgaaggatttctccgttactaagctacaacgggcaagaagctcaatgtacggttcaaac
     L  K  D  F  S  V  T  K  L  Q  R  A  R  S  S  M  Y  G  S  N  94
282 tacaacggcaaggactatggttatatggtctttgacggtgtcagcgacttctatcaactt
     Y  N  G  K  D  Y  G  Y  M  V  F  D  G  V  S  D  F  Y  Q  L  114
342 gtccggagcatcccaagaccgccaccagcttcaaacgctgtagcccccaccgtcttctac
     V  R  S  I  P  R  P  P  P  A  S  N  A  V  A  P  T  V  F  Y  134
402 tttgctcccaagaaggtttgggttcttgcgtaccagtggggtcctaccactttcacctac
     F  A  P  K  K  V  W  V  L  A  Y  Q  W  G  P  T  T  F  T  Y  154
462 cgcacatcaaccgacccctccaacgcaaactcatggggagcggcccagcccctcttctgt
     R  T  S  T  D  P  S  N  A  N  S  W  G  A  A  Q  P  L  F  C  174
```

arabinosidase III) and is to be submitted to the Genbank database. The clone is 1218 bp in size and encodes a predicted protein of 391 amino acids.

The nucleotide sequence contains a long 5' UTR of 978 bp with the following features: a capping site motif CCATTC at –161, a transcription initiation motif CAAT at –96, a Shine Delgarno sequence GGAG at –14 and the start codon located in the Kozak consensus sequence GCAATGGC, identified in *N. crassa* as the place of translation initiation. The 3' UTR contains the polyadenylation signal consensus AATAAA located 13 bp upstream of the poly (A) addition site.

Computer analysis of the deduced protein indicates a molecular weight of 43,123 kDa and a predicted isoelectric point of 9.0. The sequence shows high probability of a signal peptide of 48 residues, being the most likely cleavage site between positions 48 and 49. The protein appears to be cytoplasmic (P= 0.52).

Predicted arabinosidases were subjected to analysis by BLAST using Fasta 3 searching for similarities. The three cDNA clones matched with other arabinosidases belonging to the glycosyl hydrolase family 62 as shown in Fig 49.

Alignments of the xylanases and arabinosidases with wide-ranging fungal and bacterial amino acid sequences were produced using the GCG alignment program, which were then used to construct unrooted trees by a distance method (Tajima and Nei algorithm) within the Treecon software package. The resultant dendrograms are shown in Fig 50 and Fig 51 for xylanase and arabinosidase respectively. In both dendrograms, sequences form three discrete groups supported by high bootstrap values.

Xylanases (Fig 50) can be described as containing two groups (I and III) of mainly fungal sequences and the other one (group II) of a mixture of bacterial and fungal sequences, separated with high bootstrap values. Within group I *Humicola grisea*, *H. insolens* and SncXYI comprise one subgroup while *Hypocrea jecorina* and *Trichoderma reesei* make the other subgroup. To these subgroups the other xylanases from the fungi *Magnaporthe*

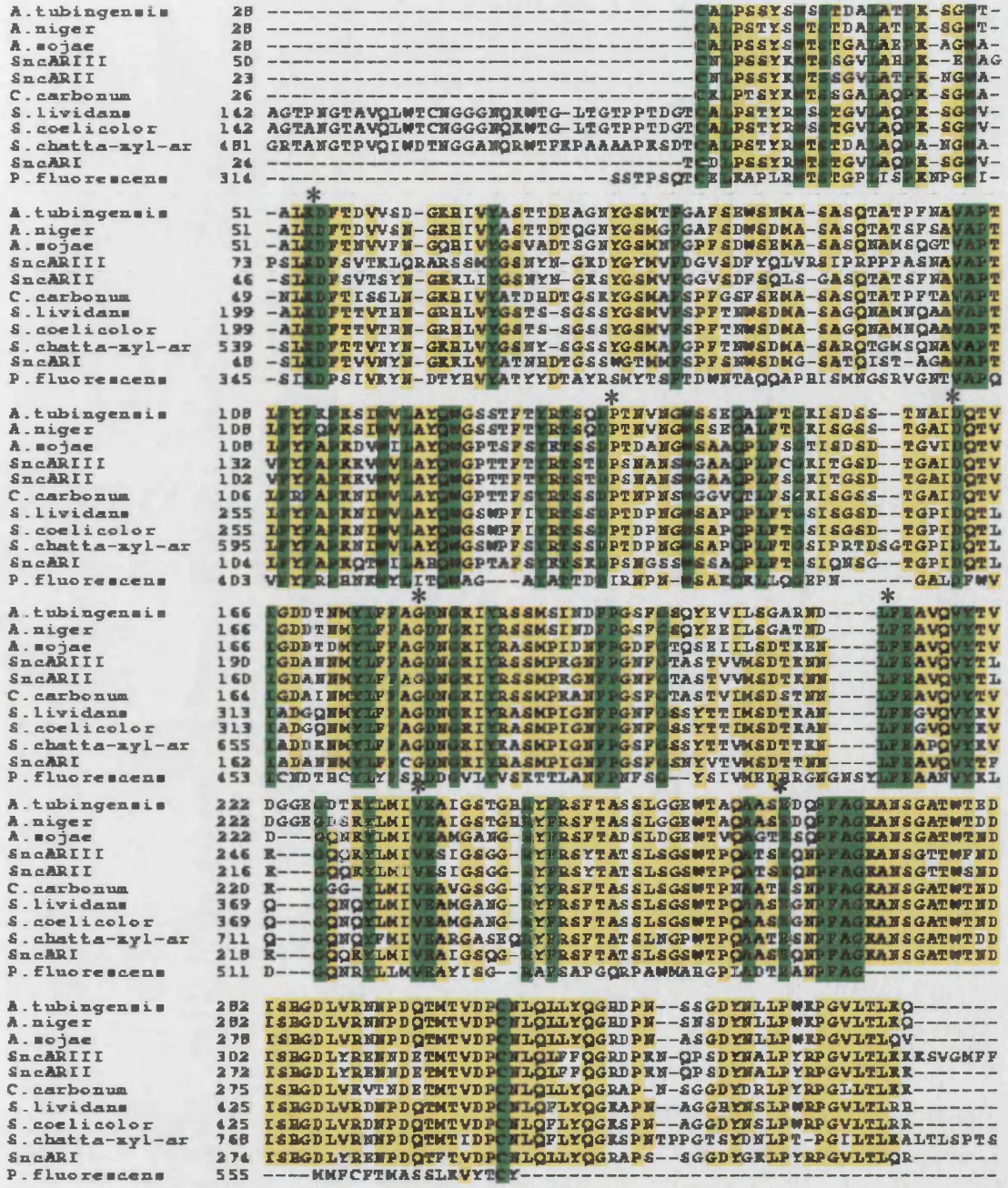


Figure 49 : Multiple protein sequence alignment showing that **SncAR I, II** and **III** belong to Family 62 glycosyl hydrolases, together with arabinosidases from: *Aspergillus tubingensis, A. niger, A. sojae, Cochliobolus carbonum, Streptomyces lividans, S. coelicolor, S. chattanoogensis* and *Pseudomonas fluorescens*. Positions of the amino acids in the primary sequences are shown in the numbers at the left of the blocks. Amino acid residues that exhibit identity in all proteins are coloured in green, residues with 60 to 80% similarity are coloured in yellow. Asterisks show conserved aspartate and glutamate residues.

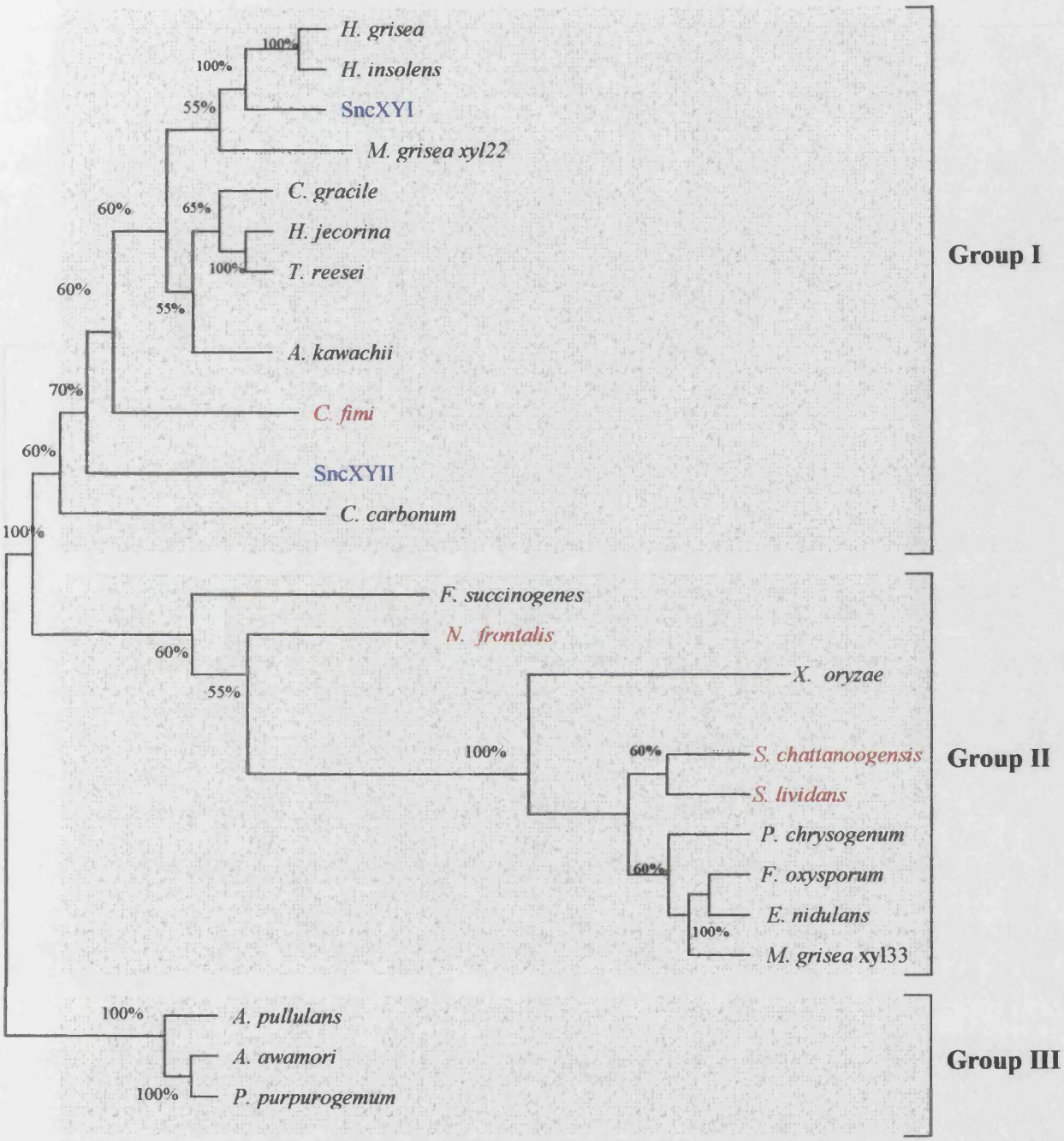


Figure 50 : Unrooted tree by a distance method of diverse xylanase sequences, produced by Treecon package software. Branch lengths are proportional to distances between sequences. Percentages indicate bootstrap values or probability that the group below is correctly assigned. Bifunctional xylanases (xylanase-arabinosidase) or xylanases being part of multienzymatic complexes are highlighted in red. *S. nodorum* xylanases are highlighted in blue.

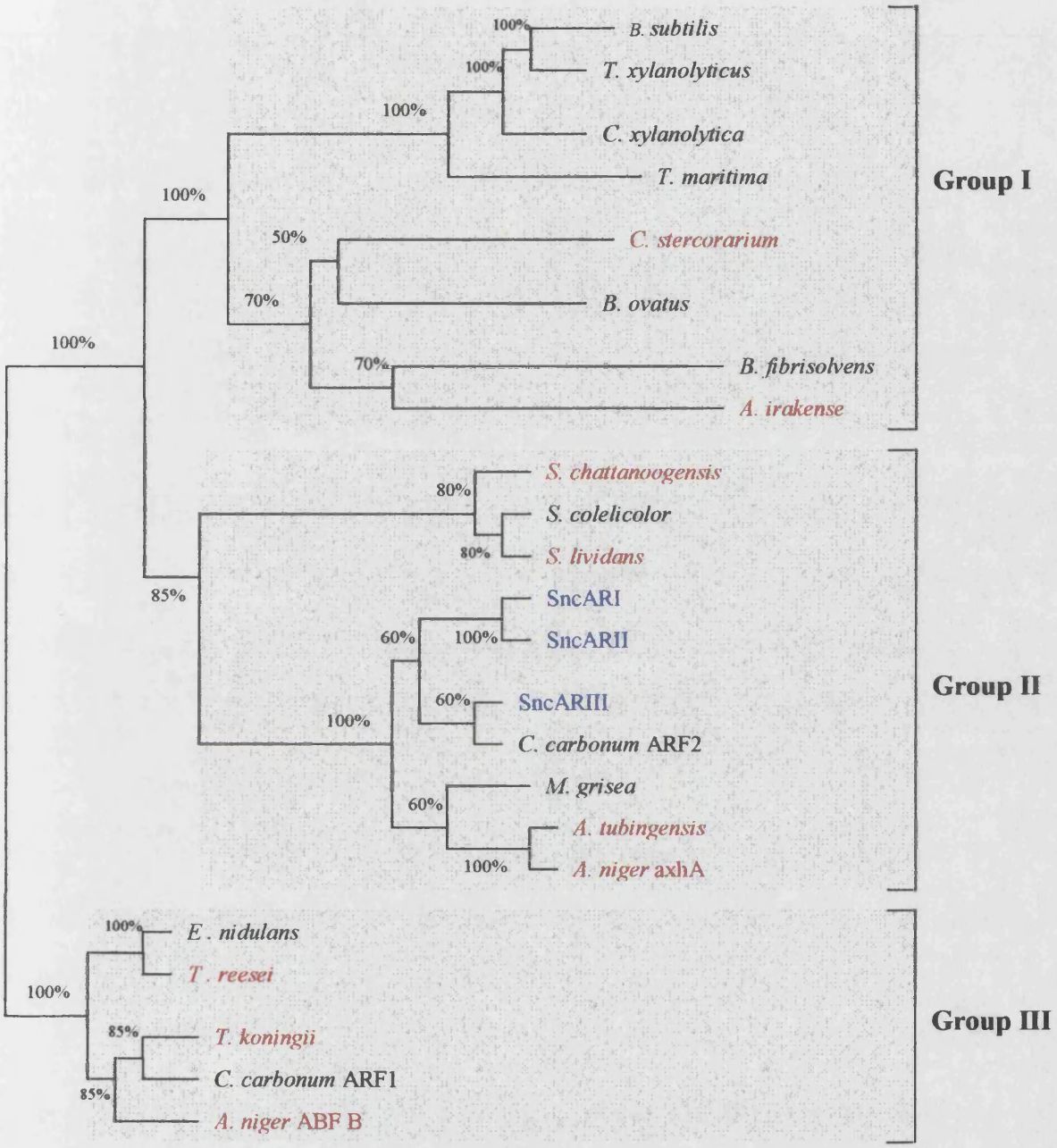


FIGURE 51 : Unrooted tree by a distance method of diverse arabinosidase sequences, produced by Treecon package software. Branch lengths are proportional to distances between sequences. Percentages indicate bootstrap values or probability that the group below is correctly assigned. Bifunctional arabinosidases (xylanase-arabinosidase) or arabinosidases localized within multienzymatic complexes are highlighted in red. *S. nodorum* arabinosidases are highlighted in blue.

grisea, *Chaetomium gracile* and *Aspergillus kawachii* grouped with low bootstrap values; then *Cellulomonas fimi*, the only bacterial xylanase and the only within a multienzymatic complex in group I, join with SncXYII and *Cochliobolus carbonum* xylanase with average bootstrap values. Group III, closely related with group I, contains xylanases from *Aureobasidium pullulans*, *A. awamori* and *Pseudomonas purpurogenum*. Finally, more distanced group II contains xylanases from *Xanthomonas oryzae*, *Streptomyces chattanoogensis*, *S. lividans*, *Penicillium chysogenum*, *Fusarium oxysporum*, *Emericella nidulans*, *M. grisea*, *Neocallimastix frontalis* and *Fibrobacter succinogenes*.

Arabinosidases (Fig 51) can be described as conforming to one group of bacterial sequences (group I), distanced from other fungal sequences (group III) and the last group of mixed sequences (group II), in between the other two groups and containing SncAR I, II and III. Group I comprises arabinosidase sequences from *Bacillus subtilis*, *Thermobacillus xylanilyticus*, *Cytophaga xylanolytica*, *Thermotoga maritima Clostridium stercorarium*, *Bacillus ovatus*, *Butyrivibrio fibrisolvens* and *Azospirillum irakense;* group III contains sequences from *Emericella nidulans*, *Trichoderma reesei*, *T. koningii*, *Cochliobolus carbonum* ARF1 and *Aspergillus niger* ABF B.

Within group II, SncARI and II make one subgroup to which SncARIII and *C. carbonum* ARF2 combine with average bootstrap value. *Aspergillus tubingensis* and *A. niger* axhA make the other subgroup to which *M. grisea* is included with average bootstrap values. Both subgroups combine with the group conformed by arabinosidase sequences from *Streptomyces coelicolor*, *S. chattanoogensis* and *S. lividans* with high bootstrap values.

4. 4 Discussion

Two xylanases and three arabinosidases were detected and isolated from a cDNA library by the use of PCR amplified probes from *S. nodorum* genomic DNA. Since the library was prepared from RNA collected from the mycelia during 16 to 40 h of culture, activation and expression of the cloned genes must occur early after contact of the fungus with the wheat cell walls. Successful screening of the library with probes designed on the basis of protein similarity shows the high degree of conservation of arabinosidase and xylanase genes among a broad spectrum of microorganisms. This result can be considered as a starting point for cloning and characterisation of putative pathogenicity determinants in this system. The final goal for cloning and characterization is gene disruption, which remains the most powerful tool for studying the role of CWDE in the virulence and pathogenicity of phytopathogenic fungi and bacteria, although in many cases gene knoutouts of putative pathogenicity determinants have not give conclusive answers (Annis and Goodwin, 1997).

In total 6234 bp of *S. nodorum* were sequenced in both strains. Translation of the nucleotide sequences revealed single open reading frames for the five cDNAs, though SncARIII seems to contain an insertion in the 5' region. However no matches were found when this long 5' extreme was blasted in the databases. The assignment of the proposed translation initiation codons was based on the following observations: (i) there are no ATG sequences upstream of the open reading frame (ORF), and (ii) translation stop codons in the ORF are all in frame with the putative translational start codon. Inspection of the nucleotide sequence in the vicinity of the putative ATG start codon did not reveal any alternative sequences which could act as translational start codons and give sequences long enough to match the enzymes.

Interpretation of Southern blots revealed that there are at least 3 xylanase genes related with the designed probe. During the alignment of xylanase sequences two

distinctive groups of similarity were made; one containing the sequences indicated in Fig 35 and used for the design of the probe, and a second group containing xylanase sequences from *Xanthomonas oryzae* pv. *oryzae*, *Streptomyces lividans*, *Fibrobacter succinogenes*, *Fusarium oxysporum* and *Emericella nidulans* (*Aspergillus nidulans*), which was not used, since positive results were obtained with the first designed probe. Similarly, during the design of arabinosidase probes two similarity groups were made. It is possible that new probes designed with the second groups would reveal more xylanase and arabinosidase genes related to the sequences within those groups and that appeared as faint bands in the Southern blot show in Fig 38.

Groups of similarity for xylanase are also confirmed in the branching pattern of the dendrogram on Fig 50, which is intended to show the relationships of xylanases from filamentous fungi (either plant pathogens or saprophytes) and bacteria, by assigning them into groups and in branch lengths that are proportional to distances between the sequences. The tree is based on amino acid comparisons for conserved regions among the proteins, and then it is expected that it will reflect somehow the classification of glycosyl hydrolases into families developed by Henrissat, in which proteins within a family are structurally related and have probably evolved from a common ancestral gene (Henrissat *et al.*, 1989). Thus, groups I and III which are more related, contain sequences mostly from family 11 while group II contains members of family 10.

Hydrophobic cluster analysis has shown that cellulases and xylanses can be grouped into nine families (Gilbert *et al.*, 1992). Xylanases can also be grouped into families based on molecular weight and pI, kinetic properties, or their crystal structure (Wong *et al.*, 1988). The IUB-MB enzyme nomenclature of a glycosyl hydrolase is based on their substrate specificity and occasionally on their molecular mechanism; such a classification does not reflect (and was not intended to) the structural features of these enzymes. A classification of glycosyl hydrolases families based on amino acid sequence similarities has been proposed a few years ago. Because there is a direct relationship between sequence and folding similarities, such a classification is expected to : (i) reflect the structural features of these enzymes better than their sole substrate specificity, (ii) help to reveal the evolutionary relationships between these

enzymes and (iii) provide a convenient tool to derive mechanistic information. Some glycosyl hydrolases are multifunctional enzymes that contain catalytic domains that belong to different families (Henrissat, 1991). On this classification, xylanases are classified mainly into family 10 and family 11 (formerly families F and G, respectively) of glycosyl hydrolases. Family 10 enzymes are larger and produce smaller oligosaccharides as end products. Family 11 xylanases are more specific for xylan, that is, they do not have cellulase or other polysaccharidase activity. Like xylanases of family 10 they have been isolated from both bacteria and fungi. They consistently have a low molecular weight and do not usually possess xylan-specific non-catalytic binding domains, which are common in family 10. The only family 11 xylanases known to have such substrate binding domains are TfxA of *Thermomonospora fusca* and XYLB of *Streptomyces lividans* (Jeffries, 1996). The deduced sequences of SncXY I and II contain residues identical to those that are characteristic of glycosyl hydrolase family 11 from fungi and bacteria (Henrissat, 1991). Glycosyl hydrolases family 11 contain two diagnostic conserved regions, which surround the two catalytic glutamate residues of these enzymes; many of the conserved residues of endo-xylanases are believed to be structurally important for correct folding and packing. On the basis of the information generated by the MOTIFS program, the residues at positions 138-148 and 229-240 (Fig 44) and 120-130 and 211-222 (Fig 45) are identical to other members of family 11 of glycosyl hydrolases as shown below:

Glycosyl Hydrolase F 11-1

(P, S) (L, Q) x E Y Y (L, I, V, M)2 (D, E) x (F, Y, W, H, N) (P) (L) x E Y Y (I,V)

SncXYI 138 : P L V E Y Y V I E S Y _ _ _ _

SncXYII 120: P L I E Y Y I V E T Y _ _ _ _

Glycosyl Hydrolase F 11-2

(L, I, V, M, F) (A,G) E (Y,W,G)2 (Q,R,F,G,S) (S,G) (S,A,N) G x (S,A) (V)

SncXYI 229 : V A T E G Y Q S S G N S _

SncXYII 211: I A T E G Y Q S S G S A _

Comparison of the amino acid sequence of SncAR I, II, and III with those of other glycosyl hydrolases indicated that the enzymes belong to glycosyl hydrolase Family 62. This family contains at present time four bacterial arabinofuranosidases, one of them that also displays xylanase activity (*Streptomyces chattanoogensis*) and 4 fungal arabinofuronasidases; among them, arabinofuranosidases from *Aspergillus tubingensis*, *A. niger* and *S. lividans* make part of multienzymatic complexes. Conserved domains in members of this family (Fig 49) include six conserved amino acid regions surrounding glutamate or aspartate residues as follows:

(i) (L, I) K **D** (F, P) (S, T)
(ii) Y (R , K) T (S, T) (Q, S, T, K, D) **D** (P, I) (S, T, R) (N, D)
(iii) (G, N) (A, V, P) (I, L) **D** (Q, F) (T, W) (L, V) I
(iv) Y L (F, Y) F (A, C, S) (G, R) **D** (N, D) G (K, V) Y
(v) L F **E** (A, G) (V, P) (Q, N) V Y (T, K)
(vi) Y (L, M, I)3 V **E** (A, S)
(vi) A (S, G, T, D, S)2 **E** (D, S, Q, G, A) (Q, N) P F A G

Enzymatic hydrolysis of the glycosidic bond takes place *via* general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base. This hydrolysis occurs *via* two major mechanisms giving rise to either an overall retention, or an inversion of anomeric configuration. The vast majority of glycosyl hydrolases contain two key catalytic carboxylic acid amino acids that mediate bond cleavage *via* general acid-base catalysis (Withers, 1995). Although the cleavage mechanism of arabinosidases is still not known, if it is proven to be by acid-base catalysis as in the rest of the glycosyl hydrolases, then it is likely that two of the four conserved aspartates and glutamates would constitute the catalytic amino acids of this enzyme.

Similarly to xylanases, the distribution of *S. nodorum* arabinosidases in the tree (Fig 51) reflects Henrissat's classification for this glycosidase. Thus, all members of Group II in the tree, in which SncAR I, II and III are located, are classified in Family 62 glycosyl hydrolases and all members of Group III belongs to Family 54 glycosyl hydrolases. Group I contain mostly members of Family 51 but two of the members,

the most distanced within the group (*B. fibrisolvens* and *A. irakense*), belong to Family 43 glycosyl hydrolases. Interestingly, two arabinosidases from *C. carbonum*, which appeared in the databases recently, belongs to two different families. The presence of SncAR I, II, and III in Group II containing bacterial and fungal enzymes supports the view that the repertoire of glycosidases expressed by a single organism is the result of extensive gene transfer, not only between different bacteria, but also between prokaryotes and lower eukaryotes. In addition, the presence of enzymes with distinct substrate specificities as is the case of members of Family 62, supports the view that subtle changes in structure of closely related proteins can lead to significant changes in catalytic specificity (Mckie *et al.*, 1997).

The deduced molecular weights and pIs of SncXYI (27.5 kDa and pI 9.2) and SncXYII (24 kDa and pI 9.0) do not appear to correspond to the xylanases forming the enzymatic complex that were detected on IEF or isolated through affinity chromatography (Chapter 3) but they match very well with the xylanase partially purified previously in our group (Xyl1) reported to be N-terminally blocked, and with a molecular weight of 30 kDa and a pI ca.10 (Carlile, 1999). Theoretical and measured pI of proteins showing discrepancy can be attributed to protein modification. It has been demonstrated that good agreement may be observed between computed (based on the primary structure) and experimental isoelectric point values when proteins of known sequence are focused under random coil (denaturing) condition. Hence, discrepancies between expected and actual data may be reliably ascribed to some kind of post-transcriptional modification, having taken place in the sample (Gianazza, 1995). During this study no basic xylanases were detected on analytical IEF gels, but only after refractionation of preparative IEF on the Rotofor (Chapter 2). Instead, aggregation appeared to occur extensively. Results of analytical IEF during deglycosylation reaction also confirm that sialic acids were the cause of pI shifting in samples run on IEF urea gels.

Perhaps SncXY I and II codify for alkaline xylanases that, however do not form part of the multienzymatic complex through non-catalytic binding domains, but aggregate with other proteins into a one single complex as in the case of *Clostridium*

papyrosolvens (Pohlschroder *et al.*, 1994), which forms an aggregation of enzymes, among them multienzymatic complexes. In *S. nodorum* the aggregation may occur through carbohydrates containing negatively charge species (sialic acid substitutions) that caused the observed smear spanning the acidic region of IEF gels and the characteristics already analysed in Chapter 3. Thus, the putative affinity purified complex is probably just a fraction of the xylanolytic system of *S. nodorum*.

The deduced amino acid sequences from the xylanase cDNAs contain the two catalytic domains characteristic of Family 11 but do not contain a cellulose-binding domain (CBD), which is prevalent in xylanases of Family 10. Considering what was discussed above, during the screening of the cDNA library the designed probe may have failed to detect (or gave faint signals) other xylanases to which it had little homology. Then, xylanases related to Family 10 glycosyl hydrolases may be present in the *S. nodorum* cDNA library, which may contain the CBD or XDB. Many xylanase genes, cDNAs and mRNAs have been reported from saprophytic and parasitic bacteria and fungi and among them, multiple xylanases with different characteristics and belonging to families 10 and 11 have been found in the same fungus, as for example *Aspergillus oryzae*, *Emericella nidulans*, *Magnaporthe grisea*, *Neocallimastrix patriciarum* and *Trichoderma reesei*. The catalytic differences between the two families suggest that they co-operate in xylan degradation, as has been observed in the *T. reseei* system (Wong *et al.*, 1988); according to these authors, xylanase producers often contain multiple loci encoding overlapping xylanolytic functions. It is well established that xylanolytic micro-organisms produce isoenzymatic xylanases that are encode by multigene families and furthermore that sequence conservation within catalytic domains of xyalanases is indicative of evolution from a small number or ancestral genes (Millward-Sadler *et al.*, 1995).

Other plant hydrolases may also contain a non-catalytic substrate-binding domain, which mediate adsorption onto insoluble substrates (Gilbert *et al.*, 1992; Ali *et al.*, 1995; Dupont *et al.*, 1998). Since the enzymatic complex of *S. nodorum* adsorbed to cell walls, insoluble xylans and cross-linked substrates, the presence of non-catalytic complex modules (CBD or XBD) is expected in any of the enzymes forming the

complex, but apparently this seems not to be the case for the isolated cDNA clones. Still, in some cellulase systems of organisms such as *Pseudomonas fluorescens* subsp *cellulosa*, *Clostridium thermocellum* among others, the majority of cellulases and hemicelluloses of the complex do not carry CBD and the affinity of these multiprotein complexes for the substrates is mediated by non-catalytic scaffolding proteins (Ali *et al.*, 1995). In the classical example of the *C. thermocellum* system, the scaffolding protein is a non-enzymatic 210 kDa glycopeptide. A similar glycosylated subunit has been described in others strains of *C. thermocellum*. The deduced primary structure of this protein contains 8 repeated domains of around 147 residues. The association of cellulases and xylanases with the scaffolding protein (SP) is mediated by an interaction between a region of duplicated sequences in the enzyme (docking domain or dockerin) and the reiterated 147-residue domains of the SP, known as cohesion domains (Poole *et al.*, 1992). Similarly, a new specific xylan–binding domain was reported for an arabinosidase of *S. lividans* (Vincent *et al.*, 1997), which is located in the N-terminal region of AbfB; the preferred substrate of this enzyme was the arabinoxylan from cereals such as oats, rye or wheat and had very low activity with the synthetic substrates *p*-nitrophenyl α-L-arabinofuranoside and 4-methylumbelliferyl α-L-arabinofuranoside.

Different combinations of the N-terminal sequences of the affinity purified complex with arabinosidase activity (Table 5 in Chapter 3) match with the translated sequences of SncAR I, II and III :

N-Terminal sequence														
S	S	P	V	W	T	S	K	D	V	L	A	N	A	D
		Y	K	E			S	K				T	S	K
							D	G					P	G

SncARI	S	S	Y	R	W	T	S	T	G	V	L	A	Q	P	K
SncARII	S	S	Y	K	W	T	S	S	G	V	L	A	T	P	K
SncARII	S	S	Y	K	W	T	S	S	G	V	L	A	H	P	K

Interestingly, the 5' ends of the amino acid sequences did not match with any other protein in the databases. It is possible that *S. nodorum* arabinosidases contain a new type of substrate-binding domain, similar to that of *S. lividans* in the sense that the putative CBD or XBD would be located in the N-terminus of the protein. Furthermore, the N-terminal sequence from a putative purified xylanase (Chapter 3) gave no homology with any reported xylanases but gave high homology (90%) only with one of the subunits of a non-covalent trimeric glycosylated complex. It is also probable that the sequenced fragment corresponds to a scaffolding protein or a non-catalytic binding domain of either arabinosidase or xylanase, of which the catalytic domains were degraded by proteases present in the samples, as discussed in the last chapter.

In summary, the genes detected and sequenced using the cDNA library constructed under the experimental conditions do not necessarily represent the full complement of xylanases or arabinosidases produced by *S. nodorum* in culture or during the course of infection. Maybe insufficient positives harbouring xylanase and arabinosidase were screened or they were missed due to low or not significant homology with the probes. In fact, two groups of similarity were made for each arabinosidase and xylanase sequences from the database in order to get areas of consensus long enough for the design of primers; and just one out of the two alignment groups for each enzyme were used, leaving the other align group in which no probe was designed. Moreover, there were two positive xylanase clones (2x and 3x) detected and their DNAs excised but unfortunately no sequence could be obtained. Insertions of some DNA into bacteria may be lethal to the bacterial cells, so that the amount of target DNA for sequence could not be achieved. That could have been the case for these clones.

CHAPTER 5 GENERAL DISCUSSION AND FUTURE WORK

In order to be successful, pathogenic fungi may require degradation of the plant cell wall, which is itself a potential source of nutrients for the pathogen. Accordingly, plant pathogens seem to have adapted to the type of cell wall of their host by producing cell wall depolymerases according to the polymer composition of the host wall. CWDEs have been considered as possible pathogenicity or virulence determinants among several candidates for pathogenicity in fungi. The major obstacle to addressing the function of wall degrading enzymes has been redundancy: all of the pathogens that have been studied in detail have multiple genes for any particular enzyme activity. Thus, most fungal strains mutated in wall-degrading genes retain at least some residual activity (Tonukari *et al.*, 2000). Despite this redundancy, single genes of a particular class have been shown, in two cases, to contribute to the virulence of pathogenic fungi-particular constitutive pectinases are virulence factors for *Aspergillus flavus* on cotton bolls and *Botrytis cinerea* on tomato (Shieh *et al.*, 1997; ten Have *et al.*, 1998).

Degradation of complex polymers like those components of the plant cell wall required a battery of enzymes. In concordance, different microorganisms that degrade plant cell walls produce an array of cellulases and hemicellulases, frequently in multiprotein complexes that facilitate enormously their regulation and also their catalytic function. Xylan is an important constituent of the *Poaceae* as the complex matrix polysaccharide, containing several heterogeneous substituents and linkages. Its degradation by xylanolytic microorganisms has been demonstrated to require a battery of enzymes containing xylanases as depolymerases and several accessory enzymes removing the lateral substituents, among them arabinofuranosidases. Moreover, disruption of the *ccSNF1* gene of *C. carbonum*, which is required for expression of catabolite repression genes when glucose is limited, as is the case of plant pathogenic fungi, strongly reduced the expression of several CWDE (endo-xylanases, α-arabinosidase, β-xylosidase, exo- and endo-polygalacturonase and exo-glucanase).

The mutants grew at the same rate, as the wild type on glucose but growth was impaired on complex polysaccharide substrates such as xylan, pectin and particularly on maize cell walls and arabinose. The virulence of the mutants on maize was decreased and more interestingly, the reduction of growth of mutants for all the CWDE was stronger that would have been predicted by the degree to which the measurable enzyme activities were decreased, as compared with the results obtained by mutations in the structural genes for the enzymes themselves (Tonukari *et al.*, 2000).

Results obtained in this study demonstrated the ability of *S. nodorum* to produce an array of CWDE when grown on isolated wheat cell walls and when inoculated droplets and IWF and extracts of inoculated leaves were assayed for different enzymatic activities. The extensive range of CWDE produced reflected the heterogeneity and composition of the wheat cell wall and the possible synergistic action between these enzymes required to degrade it. Under the experimental conditions, it appeared that xylanase and arabinosidase in extracts of inoculated tissues, IWF and specially inoculated droplets, were produced by the fungus early after inoculation and in correlation with the aggressivity of three different isolates used to inoculate wheat leaves. Xylanase as depolymerase and arabinosidase as glycosidase appeared to be significant among all the CWDE assayed, due to the ability of the fungus to produce them in culture with wheat cell walls, their detection in infected tissues and inoculated droplets, and the identification of the arabinosidase and xylanase isoforms appearing concomitant with steadily increasing activities *in vivo* and *in vitro*, with the comparison with host isoforms, all this suggested that both enzymes are good candidates for pathogenicity determinants.

Ultrastructural localization of these enzymatic activities with labelled antibodies to pure xylanase and arabinosidase would constitute further evidence for the involvement of these enzymes in penetration of the host cell walls in inoculated detached leaves. Also immuno-precipitation with antibodies generated against the individual components of the xylanolytic system can be used to study the effect of inactivating individual enzymes in substrate and cell walls degradation.

However purification of proteins as single bands on SDS-PAGE was shown to be a very difficult task, since attempted separation of the enzymes revealed that *S. nodorum* secretes proteins in aggregates of multiprotein complexes. These complexes give an indication of likely synergy between CWDEs, and in particular between xylanase and arabinosidase activities. Also tenable is the synergy between arabinosidases and the major wall protein-degrading trypsin of *S. nodorum* (Carlile *et al.*, 2000) in view of the protective arabinosidase side chains that abound on cell wall extensins. Indeed, it was observed in this study that incubation for three weeks of the cell wall-bound protein fraction with the insoluble cell walls remaining (after culture of *S. nodorum* in minimal medium) led to the solubilisation of most of the 1% (w/v) cell wall suspension (data not shown).

Combination of biochemical and molecular biology techniques may help to resolve this difficulty. Expression of the enzyme genes in bacterial hosts, which are able to generate proteins without sugar moieties, may facilitate decomposition of the complex into the individual activities, which may also help to identify polypeptides with more than one enzymatic activity and to study the different subunits or domains more accurately and easily. Proteins can be also expressed individually in a yeast system, which has proven to be a good strategy for the characterization of multifunctional enzymes in *T. reesei* (Magolles-Clark *et al.*, 1996). This way it would be also feasible to study the effect of the glycan moieties on the formation of the complex(es) and the possible role of sialic acids in the binding of the complex to substrates or host cell walls. Individual proteins should be able to be combined together to reconstitute the complex in order to study its stability and even its capability to degrade host cell walls and/or to induce symptoms in comparison with the non-glycosylated enzymes.

Interestingly, recent work that showed some similarities to some of the findings in this research encouraged further studies into the xylanolytic system of *S. nodorum* when the arabinosidase AF53 of *T. reesei*, was reported to be the first eukaryotic hemicellulase to contain a XBD (Nogawa *et al.*, 1999). Its molecular mass was reduced by ammonium sulphate precipitation in acid pH or pepsin treatment. The truncated AF 35 generated in this way that resulted from removal of the C-terminal

half of AF53, lost the binding activity for xylan and therefore, it was suggested that this α-arabinofuranosidase was a modular glycosidase containing both catalytic and substrate binding domains. Unfortunately, the AF35 XBD was not isolated when the peptide was treated with pepsin and furthermore, no region homologous to a CBD or XBD and linkers was found in the deduced amino acid sequence of the isolated gene for AF53. Since the binding to xylans was dependent on the ionic strengths of the buffers used in binding experiments and since it was reported that electrostatic interactions may have significant role in binding to xylan (Tenkanen *et al.*, 1995), it was postulated that the AF 53 C-terminus without catalytic function, bound to xylan by electrostatic interaction in what constitutes a new type of XBD.

Neither typical CBD nor XBD was found in the arabinosidase cDNAs isolated from the cDNA library of *S. nodorum* in this study, indicating that perhaps this is not the way the enzymes are aggregating. The deduced amino acid sequences were found to be related with that of *S. lividans* in the tree by dustabce method (Fig 51), which has been reported to contain a new substrate-binding module unusually located in the N-terminus of the protein (Vincent *et al.*, 1997). In spite of this, the N-terminal of SncAR I, II and III showed no similarity to any other sequences in the databases and remarkably, sialic acids were detected in the putative purified protein which N-terminal sequence was found in the deduced amino acid sequences of the cDNAs. This evidence indicates that the cloned cDNA most probably contains a new type of binding domain and maybe sialic acids are implicated in the ionic interactions that play a part in the binding capabilities of the enzymes to insoluble substrates; this is likely because adsorption to different substrates of arabinosidase and xylanase activities of *S. nodorum*, was influenced by the pH and ionic strength of the buffers. If the degradation of wheat cell walls and then, xylan hydrolysis, is proven to be significant during penetration and colonisation of *S. nodorum* in wheat, it might be possible to implicate glycan moieties, namely sialic acids, as determinants in the pathogenicity of a plant pathogenic fungus, as they have been already implicated in pathogenicity of human pathogenic fungi (Oda *et al.* 1983; Souza *et al.*, 1986; Rodrigues *et al.* 1997). However, there is a possibility that enzymes associate through

a third protein, the scaffolding type protein, that might be related the synovial type protein.

Another interesting experiment that can be carried out, once a system to produce pure enzymes become available, is the use of inhibitors of xylanases to test the effect of such molecules in the protection of host cell walls against fungal hydrolysis. A novel protein that can inhibit the activity of Family 11 endo-β-1,4-xylanases (like those isolated and sequenced from the *S. nodorum* cDNA library) has already been purified from wheat (McLauchlan *et al.*, 1999). According to these authors this protein inhibitor may play a role in plant defence against hydrolytic enzymes. Work is in progress to determine if that inhibitory activity is also extended to arabinosidases or other accessory enzymes.

From what has been discussed, it can be concluded that a better system for purification and further characterisation of the enzymes involved in the hydrolysis of the host cell wall is still required in order to understand the nature of these enzymes and how they may participate together in degradation of native cell walls. Important questions are waiting for answers and these answers are dependent on the understanding of the biochemistry and gene expression during the early stages of the infection and include: How does the fungus make such extensive epiphytic growth before penetration? What triggers the fungus to penetrate after the epiphytic growth? What is the first gene to be expressed? Is there co-expression of genes? A molecular approach for screening and cloning of some xylanases and arabinosidase was proved to be a good and feasible technique that can help to answer these questions.

First it would be desirable to design new probes based on the other groups for both arabinosidase and xylanase and to screen again the cDNA library for new genes. In fact, the genes detected and sequenced using the cDNA library constructed under the experimental conditions most probably not represent the full complement of xylanses or arabinosidases produced by *S. nodorum* in culture or during the course of infection; there may be more or different mRNAs and proteins (and the genes) that are expressed during *in vivo* infection as there are some clones missed during the library

screening. All possible collection of genes should be expressed in bacterial or yeast system in order to have enough protein available for biochemical characterization as previously discussed.

The evidence to show that the genes expressed *in vivo* are the same as those expressed in culture conditions is the Northern blotting in which RNA will be probed with specific probes designed from the conserved regions of the sequenced arabinosidase and xylanase genes.

It would be also advantageous to screen a genomic library with all the probes in order to get the complete arabinosidase and xylanase genes and then study the regulatory elements in the 5' end. Design of primers in the variable 5'UTR (or non-conserved regions) of the cloned genes can be used later as probes in the cDNA and genomic libraries which will be also useful in determining if different isoforms are being expressed and which ones appear during infection by comparison with Northern blots or RT-PCR. Discovery and characterisation of such isoforms could be informative since they may be responsible for redundancy of xylanses in fungi.

Finally, to investigate further the expression of xyalanse and arabinosidase in planta, *S. nodorum* can be transformed with vectors carrying the enzyme promoters fused to GFP reporter (green fluorescent protein) as it has been done with *S. nodorum* SNP1 (Carlile *et al.*, 2000)

BIBLIOGRAPHY

AKED, J and HALL, JL. (1993). Effect of powdery mildew infection on concentrations of apoplastic sugars in pea leaves. *New Pathologist.* **123** : 283-288.

ALI, B.R.S., ZHOU, L., GRAVES, F.M., FREEDMAN, R.B., BLACK, G.W., GILBERT, H.J. and HAZLEWOOD, G.P. (1995). Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and are encoded by multigene families. *FEMS Microbiology Letters.* **125** : 15-22.

ALVIANO, C.S., TRAVASSOS, L. R. and SCHAUER, R. (1999). Sialic acids in fungi: a minireview. *Glycoconjugate Journal.* **16** : 545-554.

ANDREWS, A.T. (1981). Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications, Claredon Press, Oxford. pp 88-91.

ANNIS, S. L. and GOODWIN, P. H. (1997). Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *European Journal of Plant Pathology.* **103** : 1-14.

ASPINAL, K. (1980). Carbohydrates, structure and function. Academic Press, New York.

BACHMANN, S. L. and McCARTY, A.I. (1991). Purification and co-operative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca. Applied Environmental Microbiology.* **57**: 2121-2130.

BAILEY, M.J., BIELY,P., POUTANEN, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology.* **23**: 257-270.

BAKER, C.J., WHALEN, C.H. and BATEMAN, D.F. (1977). Xylanase from *Trichoderma pseudokoningii* and effects on isolated plant cell walls. *Phytopathology.* **67** : 1250-1258.

BAKER, C.J., WHALEN, C.H., KORMAN, R.Z., AND BATEMAN, D.F. (1979). α-L-Arabinofuranosidase from *Sclerotinia sclerotiorum*: Purification, characterization, and effects on plant cell walls and tissue. *Phytopathology.* **69** : 789-793.

BARTOLOME, B., FAULDS, C.B., KROON, P.A., WALDRON, K., GILBERT, H.J., HAZLEWOOD, G. and WILLIAMSON, G. (1997). An *Aspergillus niger* esterase (ferulis acid esterase III) and recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (XylD) release a 5-5' ferulic dehydrodimer (diferulic acid) from barley and wheat cell walls. *Applied and Environmental Microbiology.* **63** : 208-212.

BEELEY, J.G. (1985). Glycoprotein and Proteolycan Techniques. In: *Laboratory Techniques in Biochemistry and Molecular Biology.* Burdon, R.H. and van Knippenberg, P.H. (eds.). Elsevier Science Publishers B.V., Amsterdam. (Volume 16).

BELDMAN, G., OSUGA, D. and WHITAKER, J.R. (1996). Some characteristics of β–D-xylopyranosidases, α-L-arabinofuranosidases and an arabinoxylan α-L arabinofuranohydrolase from wheat bran and germinated wheat. *Journal of Cereal Science.* **23** : 169-180.

BENEDIKZ, P. W., MAPPLEDORAM, C. J. and SCOTT,P. R. (1981). A laboratory technique for screening cereals for resistance to *Septoria nodorum* using detached seedings leaves. *Transactions of the British Mycological Society.* **77** : 667-669.

BENHAMOU, N. and OUELLETE, G. B. (1987). Ultrastructural characterization of an extracellular fibrilar sheath on cells of *Ascocalyx abietina*, the scleroderris canker agent of conifers. *Canadian Journal of Botany* **65** : 154-167.

BIELY, P. (1993). Biochemical aspects of the production of microbial hemicellulases. In: *Hemicelluloses and Hemicellulases.* Coughlan, M.P and Hazlewood, G.P. (eds.). Portland Press, London. pp 29-51.

BIELY, P. (1985). Microbial xylanolytic systems. *Trends in Biotechnology.* **3** : 286-290.

BIELY, P., MACKENZIE, C.R. and SCHNEIDER, H. (1988). Production of acetylxylan esterase by *Trichoderma reesei* and *Schizophylum commune. Canadian Journal of Microbiology.* **32** : 767-772.

BIELY, P., MARKOVIC, O. and MISLOVICOVA, D. (1985). Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. *Analytical Biochemistry.* **144** : 147-151.

BIELY, P., VRŠANSKÁ, M. and KRÁTKÝ, Z. (1980). Xylan-degrading enzymes of the yeast *Cryptococcus albidus.* Identification and cellular localization. *European Journal of Biochemistry.* **108** : 313-321.

BIRD, P.M. and RIDE, J.P. (1981). The resistance of wheat to *Septoria nodorum* fungal development in relation to host lignification. *Physiological Plant Pathology.* **19** : 289-299.

BLACK, G. W., HAZLEWOOD, G. P., XUE, G., ORPIN, C. G. and GILBERT, H. J. (1994). Xylanase B from *Neocallimastrix patriciarum* contains a non-catalytic 455-residue linker sequence comprised of 57 repeats of an octapeptide. *Biochemical Journal*, **299** : 381-387.

BLACK, G. W., HAZLEWOOD, G. P., MILLWARD-SADLER, S. J., LAURIE, J. and GILBERT, H. J.(1995).A modular xylanase containing a novel non-catalytic xylan-binding domain. *Biochemistry Journal.* **307** : 191-195.

BORNEMAN, W .S., LJUNGDAHL, L .G., HARTLEY, R. D., and AKIN, D. E. (1993). Feruloyl and *p*-coumaroyl esterases from the anaerobic fungus *Neocallimaxtrix* strain MC-2: properties and functions in plant cell wall degradation. In: *Hemicelluloses and Hemicellulases.* Coughlan, M.P and Hazlewood, G.P. (eds.). Portland Press, London. pp 85-102.

BRECCIA, J. D., BAIGORI, M. D., CASTRO, G. R. and SIÑERIZ, F. (1995). Detection of endo-xylanase activities in electrophoretic gels with Congo red staining. *Biotechnology Techniques.* **9** (2) : 145-148.

BURKE, D., KAUFMAN, P., McNEIL, M., and ALBERSHEIM, P. (1974). The structure of plant cell walls. VI. A survey of the walls of suspension-cultured monocots. *Plant Physiology.* **54** : 109-115.

CARRA, J. H. and PRIVALOV, P. L. (1995). Energetics of denaturation and m values of *Staphylococcal* nuclease mutants. *Biochemistry.* **34** : 2034

CARLILE, A.J., BINDSCHEDLER, L.V., BAILEY, A.M., BOWYER, P., CLARKSON, J.M. and COOPER, R.M. (2000). Characterization of SNP1, a cell wall-degrading trypsin, produced during infection by *Stagonospora nodorum. Molecular Plant-Microbe Interactions.* **13** : 538-550.

CARLSSON, S. R. (1993). Isolation and Characterisation of Glycoproteins. In: *Glycobiology a Practical Approach.* Fukuda M and Kobata A. (eds.). IRL Press, Oxford: pp 1-26.

CARMONA, C.E., BROCHETTO-BRAGA, M.R., PIZZIRANI-KLEINER, A.A. and JORGE, J.A. (1998). Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor. FEMS Microbiology Letters.* **166** : 311-315.

CARPITA, N. C. and GIBEAUT, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal.* **31** (1) : 1-50.

CATEN, C. E. and NEWTON, A. C. (2000). Variation in cultural characteristics, pathogenicity, vegetative compatibility and electrophoretic kariotype within field populations of *Stagonospora nodorum. Plant Pathology.* **49** : 219-226.

CAVENDON, K., LESCHINE, S., and CANALE-PAROLA, E. (1990a). Cellulase system of a free-living, mesophylic clostridium. *Journal of Bacteriology.* **172** : 4222-44230

CAVENDON, K., LESCHINE, S., and CANALE-PAROLA, E. (1990b). Characterization of the extracellular cellulase from a mesophylic clostridium (strain C7). *Journal of Bacteriology.* **172** : 44231-4237

COGGINS, J. R., von DOHREN, H., FRIEDRICH, P., OERHAM, P. A., SRERE, P. A. and WELCH, G. R. (1992). *Biochemical Nomenclature and Related Documents*, 2nd edition, Portland Press.

COLEMAN, M., HENRICOT, B., ARNAU, J., and OLIVER, R. P. (1997). Starvation-Induced genes of the tomato pathogen *Clamidosporium fulvum* are also induced during growth *in planta. Molecular Plant-microbe Interactions.* **10** : 1106-1109.

COLLMER, A. and KEEN, N.T. (1986). The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology.* **24** : 383-409.

COOPER, R.M. and WOOD, R. (1975). Regulation of synthesis of cell wall degrading enzymes by *Verticillum albo-atrum* and *Fusarium oxysporum* f.sp. lycopersici. *Physiological Plant Pathology.* **5** : 135-156.

COOPER, R.M. and WOOD, R.K.S. (1980). Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endo-pectin lyase in *Verticillium* wilt of tomato. *Physiological Plant Pathology.* **16** : 285-300.

COOPER, R.M. (1983). The mechanisms and significance of enzymatic degradation of host cell walls by parasites. In: *Biochemical Plant Pathology.* J.A. Callow (ed.). John Wiley & Son Ltd, New York. pp 101-135.

COOPER, R.M. (1984). The role of cell wall-degrading enzymes in infection and damage. In: *Plant Diseases: Infection, Damage and Loss.* Wood R.K.S. & Jellis G.J. (eds.). Blackwell Scientific Publications, Oxford. pp 13-27.

COOPER, R.M., LONGMAN, D., CAMPBELL, A., HENRY, M. and LEES, P.E. (1988). Enzymatic adaptation of cereal pathogens to the monocotyledonous primary wall. *Physiological and Molecular Plant Pathology.* **32** : 33-47.

COUGHLAN, M.P., TUOHY, M.G., FILHO, E.X.F., PULS, J., CLAEYSSENS, M., VRSANSKA, M., and HUGHES, M.H. (1993). Enzymologycal aspects of microbial hemicellulases with emphasis on fungal systems. In: *Hemicelluloses and Hemicellulases.* Coughlan, M.P and Hazlewood, G.P. (eds.). Portland Press, London. pp 53-84.

CREIGHTON, T.E. (1996). Proteins structures and molecular properties. W.H. Freeman and Company, New York

CREIGHTON, T.E. (1979). Electrophoretic analysis of the unfolding of proteins by urea. *Journal of Molecular Biology.* **129** : 235-264.

DANCER, J., DANIELS, A. and COOLEY, N. (1997). *Septoria tritici* and *Stagonospora nodorum* as a model pathogens for fungicide discovery. In: *15th Long Ashton International Symposium*, 1997. IACR-Long Ashton : pp 30.

DEAN, R.A. (1997). Signal pathways and appresorium morphogenesis. *Annal Review of Phytopathology*. **35** : 211 - 34

DEBEIRE, P., PRIEM, B. STRECKER, G. AND VIGNON, M. (1990). Purification and properties of an endo-1,4-xylanase excreted by a hydrolytic thermophilic anaerobe, *Clostridium thermolacticum. European Journal of Biochemistry.* **187** : 573-580.

DEBYSER, W., DERDELINCKX, G. and DELCOUR, J.A. (1997). Arabinoxylan and arabinoxylan hydrolysing activities in barley malts and worts derived from them. *Journal of Cereal Science*. **26** : 67-74.

DEISING, H., NICHOLSON, R., HAUG, M., HOWWARD, R., and MENDGEN, K. (1992). Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. *The Plant Cell* . **4** : 1101-1111

DESHPANDE, V., LACHKE, C., MISHRA, S., KESKAR, S., and RAO, M. (1986). Mode of action and properties of xylanase and β-xylosidase from *Neurospora crassa. Biotechnology Bioengineering*. **28** : 1832-1837.

DEUTSCHER, M.P. (1990). Methods in Enzymology: Guide to Protein Purification. Academic Press, Inc. London. (Volume 182).

DEWEY, F.M., WONG, Y.L., SEERY, R., HOLLINS, and GURR, S.J. (1999) Bacteria associated with *Stagonospora nodorum* increase pathogenicity of the fungus. *New Phytologist*. **144** : 489-497

DICMAN, M. POLDILA, G. and KOLATTUKUDY, P. (1989). Insertion of cutinase gene into a wound pathogen enable it to infect host. *Nature*. **342** : 446-448.

DOBOZI, M. S., SZAKACS, G. and BRUSCHI, C. V. (1992). Xylanase activity of *Phanerochaete chrysosporium. Applied and Environmental Microbiology*. **58** : 3466-3471.

DOERNER, K.C. and WHITE, B.A. (1990). Detection of glycoproteins separated by non-denaturing polyacrylamide gel electrophoresis using the periodic acid-Schiff stain. *Analytical Biochemistry*. **187** : 147-150.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. and SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. **28** (3) : 350-356.

DUNN, M.J. (1993). Gel Electrophoresis: Proteins. In : The Introduction to Biotechniques series. Graham, J.M. and Billington, D. (eds.). Bios Scientific Publishers Limited, Oxford. 115-127.

DUPONT, C., ROBERGE, M., SHARECK, F., MOROSOLI, R. and KLUEPFEL, D. (1998). Substrate binding domains of glycanases from *Streptomyces lividans*: characterization of a new family of xylan-binding domains. *Biochemistry Journal.* **330**: 41-45.

DURCHSCHLAG, H. and JAENICKE, R. (1997). SDS-Electrophoresis of glycoproteins. *CHIMICA OGCI-Chemistry Today.* **15** (9-10) : 15-24.

EDGE, A.S.B., FALTYNEK, C.R., HOF, L., REICHERT Jr., L.E. and WEBER, P. (1981). Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Analytical Biochemistry.* **118** : 131-137.

ESQUERRE-TUGAYE, M.T., BOUDART, G. and DUMAS, B. (2000). Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. *Plant Physiological Biochemistry.* **38** : 157-163.

FAIRBANKS, G., STECK, T.L. and WALLACH, D.F.H. (1971). Electrophoresis analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10** (13) : 2606-2616.

FERNÁNDEZ-ESPINAR, M.T., PEÑA, J.L., PIÑAGA, F. and VALLÉS, S. (1994). α-L arabinofuranosidase production by *Aspergillus nidulans*. *FEMS Microbiology Letters.* **115** : 107-112.

FILHO, E.X.F., PULS, J. and COUGHLAN, M.P. (1996). Purification and characterization of two arabinofuranosidases from solid-state cultures of the fungus *Penicillium capsulatum*. *Applied and Environmental Microbiology.* **62** (1) : 168-173.

FILLINGHAM, I.J., KROON, P.A., WILLIAMSON, G., GILBERT, H.J. and HAZLEWOOD, G.P. (1999). A modular cinnamoyl ester hydrolase from the anaerobic fungus *Piromyces equi* acts synergistically with xylanase and is part of a multiprotein cellulose-binding cellulase-hemicellulase complex. *Biochemistry Journal.* **343** : 215-224.

FITCHETTE-LAINÉ, A.C., DENMAT, L.A., LEROUGE, P. and FAYE, L. (1998). Analysis of *N*- and *O*-glycosylation of plant proteins. In: *Methods of Biotechnology (Volume 3) : Recombinant Proteins From Plants: Production And Isolation Of Clinically Useful Compounds*. Cunningham, C. and Porter, A.J.R.. (eds.). Humana Press Inc., Totawa, New Jersey. 271-290.

FLIPPHI, M.J.A., van HEUVAL, M., van der VEEN, P., VISSER, J. and de GRAFF, L.H. (1993). Cloning and characterization of the *abf*B gene coding for the major α-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. *Current Genetics.* **24** : 525-532.

FRY, S.C. (1998). The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Longman Scientific & Technical.

FUJI, T., SUZUKI, T., and KATO, R. (1981). Effect of a growth inhibitor on the hydroxyproline level in cell wall of Zea primary roots. *Plant Cell Physiology.* **22**: 1185 - 1190.

GAO, S., CHOI, G., SHAIN, L. and NUSS, D. (1996). Cloning and targeted disruption of enpg-1 encoding the major in vitro endopolygalacturonase of the chestnut blight fungus. *Applied and Environmental Microbiology.* **62** : 1984-1990

GASPAR, A., COSSON, T., ROQUES, C., and THONART, P. (1997). Study on the production of a xylanolytic complex from *Penicillium canescens. Applied Biochemistry and Biotechnology.* **67** : 45-58.

GIANAZZA, E., GALLIANO, M. and MILLER, I. (1997). Structural transitions of human serum albumin: an investigation using electrophoretic techniques. *Electrophoresis.* **18** : 695-700.

GIANAZZA, E. (1995). Isoelectric focusing as a tool for the investigation of post-translational processing and chemical modifications of proteins. *Journal of Chromatography A.* **705** : 65-87.

GIELKENS, M.M., VISSER, J. and de GRAFF, L.H. (1997). Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus tubingensis. Current Genetics.* **31** (1) : 22-29.

GILBERT, H.J., HAZLEWOOD, G.P., LAURIE, J.I., ORPIN, C.G. and XUE, G.P. (1992). Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. *Molecular Microbiology.* **6** (15) : 2065-2072.

GILBERT, H.J., and HAZLEWOOD, G.P. (1993). Bacterial cellulases and xylanases. *Journal of General Microbiology.* **139** : 187-194.

GILKES, N.R., HENRISSAT, B., KILBURN, D.G., MILLER, R. C., and WARREN, R.A.J. (1991). Domains in microbial β-1,4-glycanase : sequence, conservation, function, and enzyme families. *Microbiological Review.* **55** : 303-315.

GLOSSMANN, H. and NEVILLE Jr., D.M. (1971). Glycoproteins of cell surfaces: A comparative study of three different cell surfaces of the rat. *The Journal of Biological Chemistry.* **246** (20) : 6339-6346.

GOMES DE SEGURA, B. and FEVRE, M. (1993). Purification and characterization of two 1,4-β-xylan endohydrolases from the rumen fungus *Neocallimastrix frontalis. Applied and Environmental Microbiology.* **59** : 3654-3660.

GORBUNOFF, M.J. (1980). Purification of ovomucoid by hydroxyapatite chromatography. *Journal of Chromatography.* **187** : 224-228.

GREVE, J.C., LABAVITCH, J. M., and HUNGATE, R.E. (1984). α-L-arabinofuranosidase from *Ruminococcus albus* 8: purification and possible role in hydrolysis of alfalfa cell wall. *Applied Environmental Microbiology.* **47** : 1135-1140.

HAMMERSCHMIDT, R., LAMPORT, D., AND MULDOON, E. (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum. Physiological Plant Pathology.* **24 :** 43-47.

HAZLEWOOD, G.P. and GILBERT, H.P. (1993). Molecular Biology of Hemicellulases. In : *Hemicelluloses and Hemicellulases.* Coughlan, M.P and Hazlewood, G.P. (eds.). Portland Press, London. pp 103-126.

HE, L., BICKERSTAFF, G.F., PATERSON, A. and BUSWELL, J.A. (1993). Purification and partial characterization of two xylanases that differ in hydrolysis of soluble and insoluble xylan fractions. *Enzyme and Microbial Technology.* **15** (1) : 13-18.

HENRISSAT, B. (1992). Analysis of Hemicellulases Sequences. Relationships to other Glycanases. In: *Xylans and Xylanases.* Visser, J., Beldam, G., Kusters-van Sorem, M.A., and Voragen, A.G.J. (eds.). Elsevier, Amsterdam. pp 97-110.

HENRISSAT, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal.* **280** : 309-316.

HENSEL, M. and HOLDEN, D.W. (1996). Molecular genetic approaches for the study of virulence in both pathogenic bacteria and fungi. *Microbiology.* **142** : 1049-1058.

HOLDEN, F.R. and WALTON, J.D. (1992). Xylanases from the fungal maize pathogen *Cochliobolus carbonum. Physiological and Molecular Plant Pathology.* **40** : 39-47.

HOWELL, H.E. (1975). Correlation of virulence with secretion in vitro of three wal-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. *Journal of General Microbiology.* **90** : 32-40

IMPERIALI, A. and O'CONNOR, S.E. (1999). Effect of N-linked glycosilation on glycopeptide and glycoprotein structure. *Current Opinon in Chemical Biology.* **3** : 643-649

ITO, K., OGASAWARA, H., SUGIMOTO, T., and ISHIKAWA, T. (1992). Purification and properties of acid stable xylanase from *Aspergillus kawachii. Bioscience Biotechnology and Biochemistry.* **56** : 547-550.

IRWIN, D., JUNG, D. and WILSON, D. B. (1994). Characterization of a *Thermomospora fusca* xylanase. *Applied Environmental Microbiology.* **60** : 763-770

JAISHREE, P. and VARMA, A. K. (1992). Glycoproteins components of cellulase and xylanase enzymes of a *Bacillus* sp. *Biotechnology Letters.* **14** : 207-212

JEFFRIES, T. W. (1996). Biochemistry and genetics of microbial xylanases. *Current Opinion in Biotechnology.* 7 : 337-342.

JOHANSSON, B.G. and HJERTEN, S. (1974). Electrophoresis, crossed immunoelectrophoresis, and isoelectric focusing in agarose gels with reduced electroendosmotic flow. *Analytical Biochemistry.* **59** : 200-213.

JOHNSON, D. G., SILVA, M., MACKENZIE, C., SCHNEIDER, H. and FONTANA, J. D. (1989). Microbial degradation of hemicellulosic materials. *Applied Biochemistry Biotechnology.* **20/21** : 245-258.

JORGENSEN, H.J. and SMEDEGARD, V. (1997). Host-Pathogen Interactions in the Septoria Disease Complex. In: *15Th Long Ashton International Symposium*, 1997. IACR-Long Ashton.

JOSELAU, J.P., COMTAT, J., and RUEL, K. (1992). Chemical Structure of Xylans and their Interaction in the Plant Cell Walls. In: *Xylans and Xylanases.* Visser, J., Beldam, G., Kusters-van Sorem, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam. pp 1-15.

KARJALAINEN, R (1985). Host-pathogen interaction between spring wheat and *Septoria nodorum* with reference to resistance breeding. *Journal of Agricultural Science in Finland.* **57**: 1-66.

KARJALAINEN, R. and LOUNATMAA, K. (1986). Ultrastructure of penetration and colonization of wheat leaves by *Septoria nodorum. Physiological and Molecular Plant Pathology.* **29** : 263-270.

KELLER, B., WINZELLER, H., WINZELLER, M., and FRIED, M.P. (1994). Differential sensitivity of wheat embryos against extracts containing toxins of *Septoria nodorum* : first step towards *in vitro* selection. *Journal of Phytopathology.* **141** : 233-240.

KELLETT, L., POOLE, D., FERREIRA, L., DURRANT, A., HAZLEWOOD, G., and GILBERT, H. (1990). Xylanase B and arabinofuranosidase from *Pseudomonas fluorencens* subsp.*cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. *Biochemical Journal.* **272** : 369-376.

KEN, K., WONG, Y., TAN, L.U. and SADDLER, J. (1988). Multiplicity of β-1,4-xylanase in micro-organism: functions and applications. *Microbiological Reviews.* **52** (3) : 305-317.

KEON, J.P.R., BYRDE, R.J. and COOPER, R.M. (1987). Some aspects of fungal enzymes that degrade plant cell walls. In: *Fungal Infections of Plants. Symposium of The British Mycologycal Society.* Pegg, G.P. & Ayres, P.G. (eds.). Cambridge University Press, Cambridge. 133-157.

KIMURA, I., SASAHARA, H. and TAJIMA, S. (1995). Purification and characterization of two xylanases and an arabinofuranosidase from *Aspergillus sojae. Journal of Fermentation and Bioengineering.* **80** (4) : 33-339.

KING, J.E., COOK, R.J. and MELVILLE, S.C. (1983). A review of *Septoria nodorum* diseases of wheat and barley. *Annals of Applied Biology.* **103** : 345-373.

KISHINO, S. and MIYAZAKI, K. (1997). Separation methods for glycoprotein analysis and preparation. *Journal of Chromatography B.* **699** : 371-381.

KLEIN, A. and ROUSSEL, P. (1998). *O*-Acetylation of sialic acids. *Biochimie.* **80**: 49-57.

KOLATTUKUDY, P.E. and CRAWFORD, M.S. (1987). The role of polymer degrading enzymes in fungal pathogenesis. In: *Molecular Determination of Plant Diseases.* S. Nishimura *et al.* (eds.). Japan Sci.Soc.Press, Tokyo/Springer-Verlag, Berlin. pp 75-95.

KORMELINK, F.J.M., SEARLE-VAN LEEUWEN, W.J.F., WOOD, T.M. and VORAGEN, A.G. (1991). Purification and characterisation of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori. Applied Microbiology Biotechnology.* **35** : 753-758.

KRESL, J. J., POTEMPA, L. A. and ANDERSON, B. E. (1998). Conversion of native oligomeric to a modified monomeric form of human C-reactive protein. *The International Journal of Biochemistry and Cell Biology.* 1415-1426

KRISTUFEK, D., HODITS, R. and KUBICEK, C.P. (1994). Coinduction of α-L-arabinosidase and α-D-galactosidase formation in *Trichoderma reesei* RUT C-30. *FEMS Microbiology Letters.* **115** : 259-264.

KRUPINSKY, J. (1997a). Aggressiveness of *Stagonospora nodorum* isolates from perennial grasses on wheat. *Plan Disease.* **81** : 1032-1036.

KRUPINSKY, J. (1997b). Stability of *Stagonospora nodorum* isolates from perennial grass host after passage through wheat. *Plan Disease.* **81** :1037-1041.

KUBATA, B.K., SUZUKI, T., HORITSU, H., KAWAI, K. and TAKAMIZAWA, K. (1994). Purification and characterization of *Aeromonas caviae* ME-1 xylanase V, which produces exclusively xylobiose from xylan. *Applied and Environmental Microbiology.* **60** (2) : 531-535.

KULKARNI, N., SHENDYE, A. and RAO, M. (1999). Molecular and bitechnological aspects of xylanases. *FEMS Microbiology Reviews*. **23** : 411- 456

KUMAR, N.N. and DEOBAGKAR, D.N. (1996). Multifunctional Glucanases. *Biotechnology Advances*. **14** : 1-15.

LABORDA, F., ARCHER, S.A., FIELDING, A.H., and BYRDE, R.J. (1974). Studies on the α-L-Arabinosidase complex from *Sclerotinia fructigena* in relation to brown rot of apple. *Journal of General Microbiology*. **81** : 151-163.

LAEMMLI, U.K. (1970). Cleavege of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** : 680-685.

LEBRUN-FOURCY, C., RONDOT, J. REVOL, C. and RENVERSEZ, J.C. (1996). L'isoélectrofocalisation sur gel d'agarose: application à l'étude des anomalies de clonalitédes immunoglobulines dans le LCR et le sérum. *Annales de Biologie Clinique*. **54** (7) : 289-292

LEHTINEN, U. (1993). Plant cell wall degrading enzymes of *Septoria nodorum*. *Physiological and Molecular Plant Pathology*. **43** : 121-134.

LIENART, Y., COMTAT, J. and BARNOUD, F. (1985). Purification of cell-wall β-D-xylanases from *Acacia* cultures cells. *Plant Science*. **41** : 91-96.

LIN, L.L. and THOMSON, J.A. (1991) An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c. *FEMS Microbiology Letters*. **84** : 197-204.

LÓPEZ-FERNÁNDEZ, C.L., RODRIGUEZ, J., BALL, A.S., COPA-PATIÑO, J.L., PÉREZ-LEBLIC, M.I. and ARIAS, M.E. (1998). Application of the affinity binding of xylanases to oat-spelt xylan in the purification of endoxylanase CM-2 from *Streptomyces chattanoogensis* CECT 3336. *Applied Microbiology and Biotechnology*. **50** : 284-287.

LUONTERI, E., SIIKA-AHO, M., TENKANEN, M. and VIIKARI, L. (1995). Purification and characterization of three α-arabinosidases from *Aspergillus terreus*. *Journal of Biotechnology*. **38** : 279-291.

MACKENZIE, C.R. and WILLIAMS, R.E. (1984). Detection of cellulase and xylanase activity in isoelectric-focused gels using agar substrate gels supported on plastic film. *Can. J. Microbiol.* **30** : 1522-1525.

MAGRO, P. (1984). Production of polysaccharide-degrading enzymes by *Septoria nodorum* in culture and during pathogenesis. *Plant Science Letters*. **37** : 63-68.

MAI, V., JUERGEN, W. and LORENTZ, W. (2000). Cloning, sequencing and characterisation of the bifunctional xylosidase-arabinosidase from the anaerobic thermophile *Thermoanaerobacter ethanolicus*. *Gene*. **247** : 137-143.

MANZI, A.E. and VARKI, A. (1993). Compositional analysis of glycoproteins. In: *Glycobiology a Practical Approach.* Fukuda M and Kobata A. (eds.). IRL Press, Oxford. pp 27-76.

MARGOLLES-CLARK, M., TENKANEN, M., NAKARI-SETALA, T. and PENTTILA, M. (1996). Cloning of genes Encoding α-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae. Applied and Environmental Microbiology.* **62** : 3840-3846.

MANSFIELD, J. (1990). Recognition and response in plant/ fungus interaction .In : *Recognition and Response in Plant-Virus interaction.* Fraser R. S. S. (ed.). Springer-Verlag, Berlin. 31-52.

MATTE, A. and FORSBERG, C.W. (1992). Purification, characterisation and mode of action of endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85. *Applied Environmental Microbiology.* **56** : 157-168.

MBWAGA, A.M., MENKE, G. and GROSSMANN, F. (1997). Investigations on the activity of the cell wall-degrading enzymes in young wheat plants after infection with *Pseudocercosporella herpotrichoides* (Fron) Deighton. *Journal of Phytopathology.* **145** : 123-130.

McKIE, V.A., BLACK, G.W., MILWARD-SADLER, S.J., HAZLEWOOD. G.P., LAURIE, J.I. and GILBERT, H.J. (1997). Arabinanase A from *Pseudomonas fluorescens* subsp. *cellulosa* exhibits both an endo- and an exo-mode of action. *Biochemical Journal.* **323** : 547-555.

McLAUCHLAN, W.R., GARCIA-CONESA, M.T., WILLIAMSON, G., ROZA, M., REVESTEIN, P. and MAAT, J. (1999). A novel class of protein from wheat which inhibits xylanases. *Biochemical Journal.* **338 :** 441-446.

MERINO-TRIGO, A., SAMPEDRO, L., RODRIGUEZ-BERROCAL, F.J., MATO, S. and de la CADENA, M.P. (1999). Activity and partial characterisation of xylanolytic enzymes in the earthworm *Eisenia andrei* fed on organic wastes. *Soil Biology and Biochemistry.* **31 :** 1735-1740.

MILLWARD-SADLER, S. J., DAVIDSON, K., HAZLEWOOD, G. P., BLACK, G. W., GILBERT, H. J., and CLARKE, J. H. (1995). Novel cellulose-binding domains, NodB homologues and conserved modular architecture in xylanases from the aerobic soil bacteria *Pseudomonas fluorescens* subsp. *cellulose* and *Cellvibrio mixtus. Biochemical Journal.* **312 :** 39-48.

MILLWARD-SADLER, S. J., POOLE, D.M., HENRISSAT, J. H., HAZLEWOOD, G. P. and GILBERT, H. J. (1994). Evidence for general role for high-affinity non-catalytic cellulose binding domains in microbial plant cell wall hydrolases. *Molecular Microbiology.* **11:** 375-382.

MOHAND-OUSSAID, O., PAYOT, S., GUEDON, E., GELHAYE, E., YOUYOU, A., and PETITDEMANGE, H. (1999). The extracellular xylan degrading system in *Clostridium cellulolyticum* cultivated on xylan: evidence for cell-free cellulosome production. *Journal of Bacteriology.* **181** : 4035-4040.

MØLLER H.J., HEINEGÅRD, D. and POULSEN J.H. (1993). Combined Alcian blue and silver staining of subnanogram quantities of proteoglycans and glycosaminoglycans in sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry.* **209** : 169-175.

MØLLER H.J. and POULSEN J.H. (1995). Improved method for silver staining of glycoproteins in thin sodium dodecil sulfate polyacrylamide gels. *Analytical Biochemistry.* **226** : 371-374.

MONTI, R., TERENZI, H.F. and JORGE, J.A. (1991). Purification and properties of an extracellular xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea. Canadian Journal of Microbiology.* **37** : 675-681.

MORALES, P., MADARRO, A., FLORS, A. SENDRA, J.M. and PÉREZ-GONZÁLEZ, J.A. (1995). Purification and characterization of a xylanase and an arabinofuranosidase from *Bacillus polymyxa. Enzyme and Microbial Technology.* **17** : 424-429.

MORRIS, D.D., GIBBS, M.D., CHIN, C.W., KOH, M.H., WONG, K.K., ALLISON, R.W. NELSON, P.J. and BERQUIST, P.I. (1998). Cloning of the xynB gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. *Applied and Environmental Microbiology.* **64** (5) : 1759-1765.

NAIM, H., STERCHI, E. and LENTZE, M. (1988). Structure, biosynthesis, and glycosylation of human small intestinal matase-glucoamilase. *Journal of Biological Chemistry* **263** (36) : 19709-19717.

NANMORI, T., WATANABE, T., SHINKE, R., KOHNO, A. and KAWAMURA, Y. (1990). Purification and properties of thermostable xyalnase and β-xylosodase produced by newly isolated *Bacillus stearothermophilus* strain. *Journal of Bacteriology.* **172**: 6669-6672.

NEVILLE Jr., D.M. (1971). Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *The Journal of Biological Chemistry.* **246** (20) : 6328-6334.

NEWTON, A.C. and CATEN, C.E. (1988). Auxotrophic mutants of *Septoria nodorum* isolated by direct screening and selection for resistance to chlorate. *Transactions of the British mycological Society.* **90** (2) : 199- 207.

NISHITANI, K. and NEVINS, D.J. (1991). Glucuronoxylan xylanohydrolase. *The Journal of Biological Chemistry.* **266** : 6539-6543.

NOGAWA, M., YATSUI, K., TOMIOKA, A., OKADA, H. and MORIKAWA, Y. (1999). An α-L-arabinofuranosidase from *Trichoderma reesei* containing a noncatalytic xylan-binding domain. *Applied and Environmental Microbiology.* **65**: 3964-3968.

ODA L. M., KUBELKA, C. F., ALVIANO, C. S., TRAVASSOS, L. R. (1983). Ingestion of yeast forms of *Sporothrix schenckii* by mouse peritoneal macrophages. *Infect Immun.* **39** : 497-504.

OLIVER, R and OSBOURN, A. (1995). Molecular dissection of fungal phytopathogenicity. *Microbiology.* **141** : 1-9

OSBOURN, A.E., SCOTT, P.R. and CATEN, C.E. (1986). The effect of host passing on the adaptation of *Septoria nodorum* to wheat or barley. *Plant Pathology.* **35** : 135-145.

PAICE, M.G., JURASEK, L., CARPENTER, M.R. and SMILLIE, L.B. (1978). Production, characterization and partial amino acid sequence of xylanase A from *Schizophyllum commune. Applied and Environmental Microbiology.* **36** (6) : 802-808.

PANBANGRED, W., FUKUSAKI, E., EPIFANIO, E.C., SHINMYO, A. and OKADA, H. (1985). Expression of a xylanase gene of *Bacillus pumilus* in *Escherichia coli* and *Bacillus subtilis. Applied Microbiology and Biotechnology.* **22** : 259-264.

PATEL, D. (1994). Gel Electrophoresis : Essential Data. In: *Essential Data Series.* Rickwood D. and Hames B.D. (eds.). Wiley & Sons, Chichester, UK.

PAUL, J. and VARMA, A.K. (1992). Glycoprotein components of cellulase and xylanase enzymes of a *Bacillus* sp. *Biotechnology Letters.* **14** (3) : 207-212.

PELTONEN , S., KARJALAINEN, R. and NIKU-PAAVOLA, M. (1994). Purification and characterization of xylanase from *Bipolaris sorokiniana. Mycological Research.* **98** : 67-73.

PIETERSE, C. M. , DERKENSEN, A. M., and GOVERS, F. (1994a). Expression of the *Phytophtora infestans ipiB* and *ipiO* genes *in planta* and *in vitro. Mol. Gen. Genet.* **244** : 269-277

PIETERSE, C. M., VANWAET, P., VERBAKEL, H., BRASSE, P., VAN DEN BERVELTHUIS, G., and GOVERS, F. (1994b). Strcture and genomics organization of the *ipiB* and *ipiO* gene clusters of *Phytophtora infestans. Gene.* **138** : 67-77.

POHLSCHRÖDER, M., LESCHINE, S.B. and CANALE-PAROLA, E. (1994). Multicomplex cellulase-xylanase system of *Clostridium papyrosolvens* C7. *Journal of Bacteriology.* **176** (1) : 70-76.

POLLEY, J.C. and THOMAS, M.R. (1991). Surveys of disease of winter wheat in England and Wales, 1976-88. *Annuals of Applied Biology*, **119** : 1-20.

POOLE, D.M., MORAG, E., LAMED, R., BAYER, E. A., HAZLEWOOD, G.P., and GILBERT (1992). Identification of the cellulose-binding domain of the cellulosome subunit S1 from Clostridium thermocellum YS. *FEMS Microbilogical Letters* **99** : 181-186.

POUTANEN, K.(1988). An α-L-arabinofuranosidase of *Tricoderma reesei. Journal of Biotechnology.* 7 : 271-282.

POUTANEN, K. and PULS, J. (1988). Characteristics of *Tricoderma reseei* β-xylosidase and its use in the hydrolysis of solubilised xylans. *Applied Microbiology and Biotechnology.* **28** : 425-432.

POWELL, K.R., HENDLEY, J.O., POHL, K.E., FREIDBERG, A. and VOLK, W.A. (1982). Quatitation of acidic capsular polysaccharides by Alcian blue binding. *Analytical Biochemistry.* **119** : 31-37.

POWELL, L. M. and PAIN, R. H. (1992). Effects of glycosylation on the folding and stability of human, recombinant and cleaved alpha1-antitrypsin. *Journal of Molecular Biology.* **224** : 241

PULS, J., and POUTANEN, K. (1989). Mechanisms of enzymic hydrolysis of hemicelluloses (xylans) and procedures for determination of enzyme activities involved. In: *Enzyme Systems for Lignocellulose Degradation.* Coughlan, M. P. (ed.), Elsevier, London. pp 151-165.

PULS, J., SCHMIDT, O., and GRANZOW, C. (1987). α-Glucoronidase in two microbial xylanolytic systems. *Enzyme Microbiology and Technology.* **9** : 83-88.

PULS, J., POUTANEN, K., SCHMIDT, O., and LINKO, M. (1986). α-1,2-Glucorunidase in the hydrolysis of hemicellulose. In: *Proc.3rd Int. Conf. Biotechnology in the Pulp and Paper Industry.* Ericksson, K.E.E. and Ander, P. (eds.). STFI, Stockholm. pp 93-95.

RABILLOUD, T., CARPENTIER, G. and TARROUX, P. (1988). Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis.* **9** : 288-291.

RADIONOVA N.A., TAVOBILOV, I. M. and BEZBORODOV, A. M. (1983) β-Xylosidase from *Aspergillus niger* 15: purification and properties. *Journal of applied Biochemistry.* **5** : 300-312.

RANSOM, R.F. and WALTON, J.D. (1997). Purification and characterisation of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum. Carbohydrate Research.* **297** : 357-364.

REHNSTROM, A.L., FREE, S.J. and PRATT, R.G. (1994). Isolation, characterization and pathogenicity of *Sclerotinia trifoliorum* arabinofuranosidase-deficient mutants. *Physiological and Molecular Plant Pathology.* **44** : 199-206.

ROCHE, N., BERNA, P., DESGRANGES, C. and DURAND, A. (1995). Substrate use and production of α-L-arabinofuranosidase during solid-state culture of *Trichoderma reesei* on sugar beet pulp. *Enzyme and Microbial Technology.* **17** : 935-941.

RODRIGUES, M. L., ROZENTAL, S., COCEIRO, J. N., ANGLUSTER, J., ALVIANO, C. S. and TRAVASSOS, L. R. (1997). Identification of *N*-acetylneuraminic acid and its 9-*O*-acetylated derivative on the cell surface of *Cryptococcus neoformants*: Influence of fungal phagocytosis. *Infect Immun.* **65** : 143-149.

RODWELL. J.D. (1982). Heterogeneity of component bands in isoelectric focusing patterns. *Analytical Biochemistry.* **119** : 440-449.

ROZIE, H., SOMERS, W., BONTE, A., ROMBOUTS, F.M. and VISSER, J. (1992). Crosslinked xylan as an affinity adsorbent for endo-xylanases. *Carbohydrate Polymers.* **17** : 19-28.

SCHWARZ, W.H., BRONNENMEIER, K., GRÄBNITZ, F. and STAUDENBAUER, W.L. (1987). Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans. *Analytical Biochemistry.* **164** : 72-77.

SCOTT-CRAIG, J., PANACCIONE, D., CERVONE, F. and WALTON, J. (1990). Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. *The Plant Cell.* **2** : 1191-1200.

SCOTT-CRAIG, J., CHENG, Y., CERVONE, F., DE LORENZO, G., PITKIN, J. and WALTON, J. (1998). Targeted mutants of *Cochliobolus carbonum* laking the two major extracellular polygalacturonases. *Applied and Environmental Microbiology.* **64**: 1497-1503.

SHAFER, W (1994). Molecular mechanisms of fungal pathogenicity to plants. *Annual Review of Phytopathology.* **32** : 461-477.

SHAO, W. and WIEGEL, J. (1992). Purification and characterisation of a termostable β-xylosidase from *Thermoanaerobacter ethanolicus*. *Journal of Bacteriology.* **174** : 5848-5853.

SHOWALTER, A.M. (1993). Structure and function of plant cell wall proteins. *Plant Cell.* **2** : 1191-1200.

SWEIGARD, J., CHUMLEY, F., and VALENT, B. (1992). Disruption of a *Magnaporthe grisea* cutinase gene. *Molecular and General Genetics.* **232** : 183-190.

TALBOT, N.J., EBBOLE, D.J., and HAMER, J.E. (1993). Identification and characterisation of MPGI, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea. Plant Cell* . **5** : 1575-1590.

TAN, L. U., WONG, Y., and SADDLER, J. N. (1985). Functional characteristics of two D-xylanses purified from *Trichoderma harzianum. Enzyme Microbiology Technology.* 7 : 431-436.

TAN, L. U., MAYERS, P. and SADDLER, J.N. (1987). Purification and characterization of a thermostable xylanase from a thermophilic fungus *Thermoascus aurantiacus. Can. J. Microbiol.* **33** : 689-692.

TANABE, K., NISHIMURA, S., and KOHMOTO, K. (1988a). Cutinase production by *Alternaria alternata* pear pathothotype and its role in pathogenicity. *Annals of Phytopathological Society of Japan.* **54** : 483-491.

TANABE, K., NISHIMURA, S., and KOHMOTO, K. (1988b). Pathogenicity of cutinase and pectic enzymes deficient mutants of *Alternaria alternata* Japanese pear pathotype. *Annals of Phytopathological Society of Japan.* **54** : 552-555.

TANAKA, M. and UCHIDA, T. (1978). Purification and properties of α-L-arabinofuranosidase from plant *Scopolia japonica* calluses. *Biochimica et Biophysica Acta.* **522** : 531-540.

ten HAVE, A., MULDER, W., VISSER, J., and van KAN, J. A. (1998). The endopolygalacturonase gene Bcpg1 is requiered for full virulence of *Botrytis cinerea. Molecular Plant-Microbe Interactions.* **11** : 1009-1016

THOMSON, J.A. (1993). Molecular biology of xylan degradation. *FEMS Microbiology Review.* **104** : 65-82.

TONUKARI, N.J., SCOTT-CRAIG, J.S. and WALTON, J.D. (2000). The *Cochliobolus carbonum* SNF1 gene is required for cell wall-degrading enzyme expression and virulence on maize. *The Plant Cell.* **12** : 237-247.

TRIVEDI, S.M., FRONDOZA, C.G. and HUMPHREY, R.L. (1983). Modified technique for periodic acid-Schiff staining of glycoproteins on agarose-film electrophoretograms. *Clinical Chemistry.* **29** (5) : 836-838.

TUOHY,.M., PULS, J., CLAEYSSENS, M., VRSANSKAS, M. and COUGHLAN, M.P. (1993). The xylan-degrading enzyme system of *Talaromyces emersonii*: novel enzymes with activity against aryl β-xylosides and unsubstituted xylans. *Biochemistry Journal.* **290** : 515 523.

TYOHY, M.G., PULS, J., CLAEYSSENS, M., VRSANSKAS, M. and COUGHLAN, M.P. (1993). The xylan degrading enzyme system of *Talaromyces emersonii*. *Biochemical Journal*. **290** : 515-523.

VAN HOOF, A., LEKAM, H., SCHAFFER, H.J. and WALTON, J.D. (1991). A single β-1,3-glucanase secreted by the maize pathogen *Cochliobolus carbonum* acts by an exolytic mechanism. *Physiological and Molecular Plant Pathology*. **39** : 259-267.

VAN-SEUNINGEN, I. and DAVRIL, M. (1992). A rapid acid-Schiff staining procedure for the detection of glycoproteins using the PhastSystem. *Electrophoresis*. **13** : 97-99.

VIDHASEKARAN, P. (1988). Host Recognition of Pathogens and Non Pathogens In: *Physiology of Disease Resistance in Plants*. Vidhasekeran (ed.) CRC Press Inc. New York. pp 1-15.

VINCENT, P., SHARECK, F., DUPONT, C. MOROSOLI, R. and KLUEPFEL, D. (1997). New alpha-L-arabinofuranosidase produced by *Streptomyces lividans* : cloning and DNA sequence of the *abf*B gene and characterization of the enzyme. *Biochemical Journal*. **322** : 845-852.

WAIBEL, R., AMADO, R. and NEUKOM, H. (1980). Purification of an α-L-arabinofuranosidase from *Aspergillus niger* by substrate affinity chromatography. *Journal of Chromatography*. **197** : 86-91.

WALTON, J.D. (1994). Deconstructing the cell wall. *Plant Physiology*. **104** : 1113-1118.

WEBER, G.F. (1922). Septoria diseases of wheat. *Phytopathology*, **12** : 537- 558.

WEBER, K. and KUTER, D.J. (1971). Reversible denaturation of enzymes by sodium dodecyl sulfate. *The Journal of Biological Chemistry*. **246** (14) : 4504-4509.

WEGENER, S., RANSOM, R. F., and WALTON, J. D. (1999). A unique eukaryotic β-xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*. *Microbiology*. **145** (5) : 1089-1095.

WILLOUGHBY, E.W. and LAMBERT, A. (1983). A sensitive silver stain for proteins in agarose gels. *Analytical Biochemistry*. **130** : 353-358.

WILSON, C. A. and WOOD, T. M. (1992). The anaerobic fungus *Neocallimastrix frontalis*: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose. *Applied Microbiology and Biotechnology*. **37** : 125-129.

WITHERS, S. G. (1995). Catalysis in glycosyl hydrolases. In: *Carbohydrate Bioengineering.* Petersen, S. B., Svensson S. and Perdersen, S. (eds). Elsevier ScienceB. V. The Nertherlans. pp 97-113.

WONG, K.K.Y., TAN, L.U.L., and SADDLER, J.N. (1988). Multiplicity of β-1,4-xylanase in micro-organism: function and applications. *Microbiological Review.* **52** : 305-317.

WONG, K.K.Y., TAN, L.U.L., SADDLER, J.N. and YAGUCHI, M. (1986). Purification of a third distinct xylanase from the xylanolytic system of *Trichoderma harzianum. Can. J. Microbiol.* **32** : 570-576.

WU, S., KAUFFMANN, S., DARVILL, A.G. and ALBERSHEIM, P. (1995). Purification, cloning and characterization of two xylanases from *Magnaporthe grisea,* the rice blast fungus. *Molecular Plant-Microbe Interactions.* **4** : 506-514.

WYSS, D. F. and WAGNER, G. (1996). The structural role of sugars in glycoproteins. *Current Opinion in Biotechnology.* 7 : 409-416

YOSHIOKA, H., NAGATO, N., CHAVANICH, S., NILUBOL, N. and HAYASHIDA, S. (1981). Purification and properties of thermostable xylanase from *Talaromyces byssochlamydoides* YH-50. *Agricultural And Biological Chemistry.* **45** (11) : 2425-2432.

ZINK, R.T. and CHATTERJEE, A.K. (1985). Cloning and expression in *Escherichia coli* of pectinase genes of *Erwinia carotovora* subsp. *caratovora. Applied and Environmental Microbiology..***49** : 714-717.

ZYNKERNAGEL, V., RIESS, F. and WENDLAND, M. (1988). Infection structures of *Septoria nodorum* in leaves of susceptible wheat cultivars. *Journal of Plant Diseases and Protection.* **95** : 169-175.

APPENDICES

Appendix 1 :Czapek-Dox-V_8, agar and liquid media and Complete Supplement

Complete Supplement	**l^{-1}**
Difco Bacto Casamino acids	20.00 g
Difco Bacto peptone (bacteriological)	20.00 g
Yeast extract	20.00 g
Adenine-HCl	3.00 g
Biotin	0.02 g
Nicotinic acid	0.02 g
p-aminobenzoic acid	0.02 g
Pyridoxine	0.02 g
Thiamine	0.02 g
Distilled water	to make 1 L

Czapek-Dox-V_8 agar	**l^{-1}**
Czapeck-Dox agar	45.5 g
Ca CO_3	3.0 g
Oxid No 3 agar	10.0 g
V8 juice	200.0 ml
Distilled water	750.0 ml
Complete supplement	50.0 ml (added after autoclaving)

CzV8Cs Liquid medium	**l^{-1}**
Czapek-Dox liquid media	45.5 g
V8 juice (centrifugated)	200 ml
Complete supplement	50 ml
Distilled water	750 ml

Appendix 2 : Preparation of Basal Salts Liquid Media, Trace Element Solution and Extraction Buffer.

Trace elements	l^{-1}
K_2HPO_4	1.00 g
$MgSO_4.7\ H_2O$	0.50 g
$Na_2Mo\ O_4.2\ H_2O$	0.02 g
$MnCl_2.\ H_2O$	0.02 g
$FeSO_4.7\ H_2O$	0.01 g
$ZnSO_4.7\ H_2O$	0.01 g
$CuSO_4.5\ H_2O$	0.003 g

Basal salts liquid media	l^{-1}
KH_2PO_4	1.00 g
$Na\ NO_3$	2.00 g
$MgSO_4.\ 7H_2O$	0.50 g
MES (50 mM)	9.76 g
Trace elements solution	10.00 ml
adjust pH 6 using 1M NaOH	

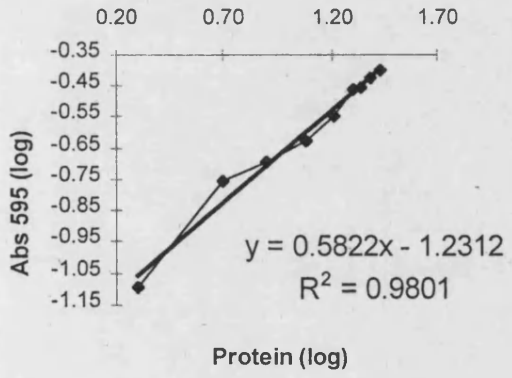
Extraction buffer	**Antiprotease Cocktail**
0.025 M sodium phosphate buffer	1 μg/ml pepstatin A
0.2 M NaCl	1 μg/ml leupeptin
5% w/v PVP (insoluble)	1 μg/ml antipain
5mM 2-Mercaptoethanol	1 μg/ml aprotinin
	0.1 mM PMSF

Appendix 3 : Preparation of Dye Reagent (Coomasie blue G, Stoscheck, 1990) and Standart Cuve for the Determination of Protein Concentration.

Dye reagent was prepare by dissolving 100 mg of Coomasie blue G (Sigma) in 100 ml 85% phosphoric acid and 50 ml 95% ethanol, then dilute to 1 L with distilled water. The reagent was kept at 4°C . When a precipitate appeared, it was decanted by centrifugation The assay was performed by adding reagents in the following order:

20µl of protein solution + 50 µl NaOH (1M) + 1 ml dye reagent

Standard Curve: BSA, TC and LY were used in concentration from 2 to 27µg . Mean of absorbances at 595 nm given by each protein at the given protein concentration is shown.



Protein (µg)	Abs 595 (nm)	Protein (log)	Abs 595 (log)
2	0.080	0.3010	-1.0969
5	0.176	0.6990	-0.7545
8	0.203	0.9031	-0.6925
11	0.204	1.0792	-0.6253
16	0.284	1.2127	-0.5467
20	0.344	1.3010	-0.4634
22	0.352	1.3377	-0.4535
24	0.377	1.3888	-0.4237
27	0.397	1.4346	-0.4012

Protein Determination : The logarithm of protein concentration vs. logarithm of mean of absorbances is plotted. The equation of the curve was used for the determination of protein concentration, by replacing in the equation the y-value (log of abs 595nm of the sample), calculating the x-value (log of concentration) and finally, calculating the antilogarithm of this number.

Appendix 4 : Somogyi-Nelson Reagents

Somogyi's Reagent I	**800 ml**
Sodium Potassium Tartrate	12g
Sodium Carbonate (anhydrous)	24g
Sodium Sulphate	144 g
MES (50 mM)	9.76 g

Somogyi's Reagent II	**200 ml**
Sodium Potassium Tartrate	12g
Sodium Carbonate (anhydrous)	24g
Copper Sulphate (5 H_2O)	4 g
Sodium Sulphate	36 g

Nelson Reagent (0.5 L)

(i) Dissolve 25 g of ammonium molybdate in 450 ml of distilled water, add 21 ml of concentrated Sulphuric acid and mix.

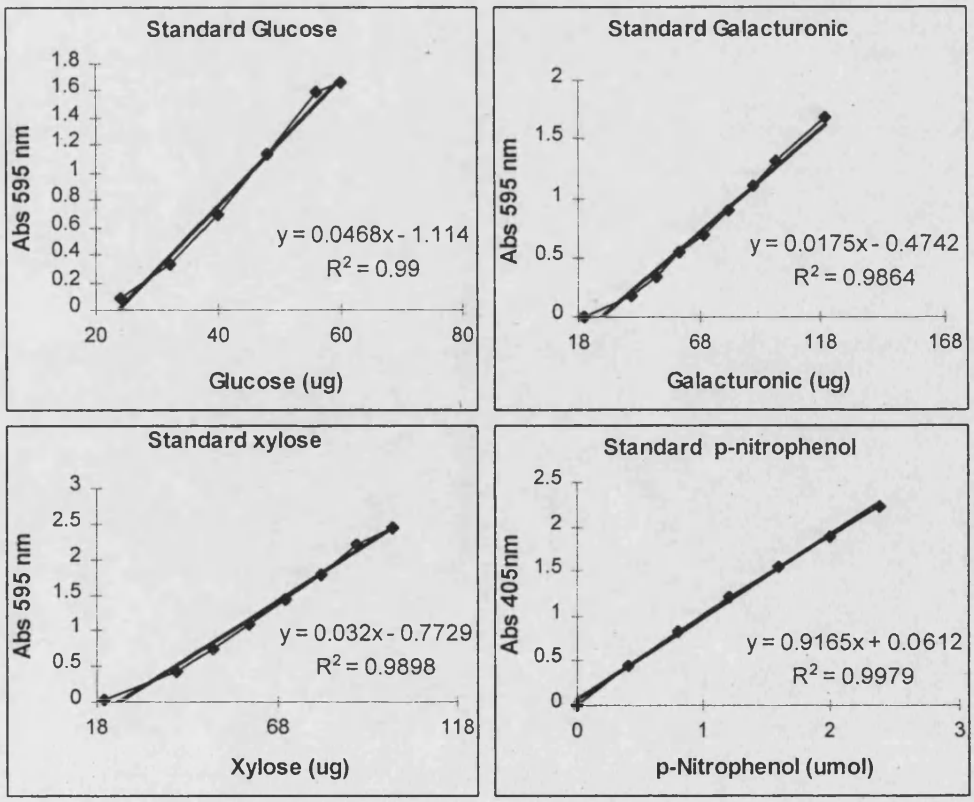
(ii) Dissolve 3 g of Na_2HAsO_4 .7 H_2O in 25 ml of distilled water.

(iii) Mix solutions (i) and (ii). Make up to 500ml with distilled water. Incubate at 37°C for 48 hours and keep in a dark bottle.

Appendix 5 : Standard Curves for the Determination of Enzymatic Activities Ex pressed as Liberated Reducing Groups (by Somoyi-Nelson) and *p*-Nitrophenol.

To express enzymatic activities as liberated µg of reducing groups, reactions were made with known concentrations of the sugar monomers glucose, galacturonic acid and xylose in the range 0-100 µg. Enzyme activities were converted to nano katals (nKats) by first transforming the absorbance at 595 mn of the reaction to be determine, to µg of reducing sugar liberated with the use the equation of the standard curve. Then, calculating the µmol/ ml and nmol/ ml per second, which is a nano katal by definition.

In a similar way, to express enzymatic activities as liberated µmol of *p*-nitrophenol, a calibration curve of *p*-nitrophenol was made in the range 0-5 µmoles. Enzyme activities were converted to nano katals (nKats) by first transforming the absorbance at 405 mn of the reaction to be determine, to µmol of p-nitrophenol liberated, then calculating nmol/ ml per second.



Appendix 6 : Gel Casting Solutions for IEF

A. Gel without additives for horizontal and vertical IEF.

Reagents	**ml**
ReadySol IEF 40% solution	1.250
MilliQ water	7.112
Ampholine	0.3 + 0.3 (accordingly with pH range)
Glycerol	1.0
Ammonium persulphate (10%)	0.035
TEMED	0.0075
Total volume solution	10

B. Gels with 6 M Urea for horizontal IEF

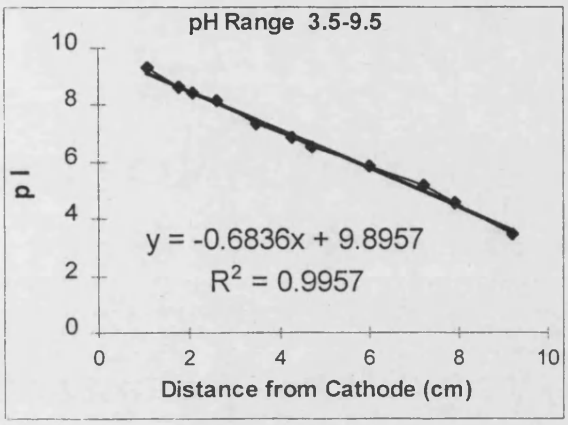
Reagents	**quantity**
Urea (6M)	3.60 g
MilliQ water	6.50 ml
Ampholytes	0.6ml + 0.6ml (accordingly with pH range)
ReadySol IEF 40% solution	1.5 ml
Ammonium persulphate (10%)	0.030 ml
TEMED	0.0075 ml
Total volume solution	10 ml

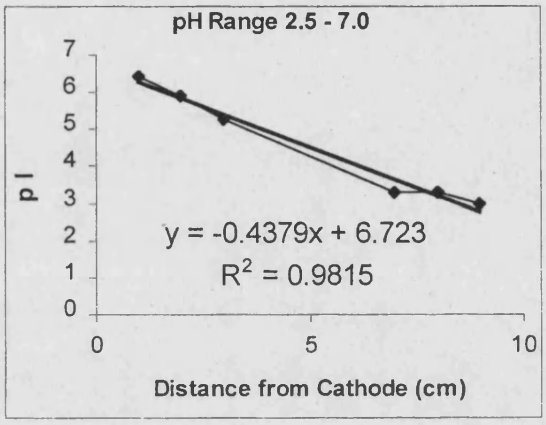
C. Composite agarose-acrylamide gels for horizontal IEF

Reagents	**quantity**
DVPM 10%	1.2 g
MilliQ water	9 ml
Agarose IEF	0.1 g
Ampholine	0.3 + 0.3 (accordingly with pH range)
Total volume solution	10 ml

Appendix 7 : Standard Curves for Determination of pI and Stock Solutions for Electrophoresis

A. Standard curves for pI calculation: Standards of known pI were isolectrofocused with samples. Distance from the cathode were measured and used to construct standard curves.





B Stock solutions for electrophoresis

Buffer 1.5 M Tris-Cl pH 8.8 Total 100 ml	18.2 g Tris base in 80 ml MilliQ water, adjust pH to 8.8 with HCl and bing to 100 ml with water. Store 4 °C.
Bufer 0.5 M Tris-Cl pH 6.8 Total 100 ml	6.1 g Tris base in 80 ml MilliQ water, adjust pH to 6.8 with HCl and bring to 100 ml with water. Store 4 °C
Sample buffer (loading buffer) 0.06 M Tris-Cl pH 6.8 Total 1000 ml.	MilliQ water 4.8 ml, buffer 0.5 M Tris-Cl pH 6.8 1.2 ml, 10% SDS 2 ml, glycerol 1 ml, 0.5%bromophenol blue (w/v water) 0.25 ml, DTT 0.50 g. Alicouts of 0.01 ml in 0.5 ml tubes kept at –70 °C, thawed and used once.
Tris-Glycine Electrode buffer stock (x 5)	15 g Tris base, 72 g glycine and 5 g SDS/ liter. Must be store in glass continer . To use dilute one part with four of water.

Appendix 8 : Polyacrylamide and Polyacrylamide Gradient Gels

A. Discontinuous buffer system for polyacrylamide gels

Reagents	Resolving gel(T10 C3.9)	Stacking gel (T4 C3.9)
MilliQ water	4.9 ml	3.17 ml
0.5M Tris-Cl pH 6.8	--------	1.25 ml
1.5M Tris-Cl pH 8.8	2.5 ml	---------
ReadySol IEF 40% solution	2.5 ml	0.5 ml
10% (w/v) SDS solution	0.1 ml	0.05 ml
10% (w/v) APS solution	0.05 ml	0.025 ml
TEMED	0.01 ml	0.01 ml
Total volume	10 ml	5 ml
To give 1 cm stacking gel	3.1 ml per slab gel was used	2 ml per slab gel was used

B. Discontinuos buffer system for polyacrylamide gradient gels (3.97-14.97% T)

Reagents	Ligth solution	Dense solution	Stacking gel
MilliQ water	0.980 ml	--------	1.295 ml
0.5M Tris-Cl pH 6.8	----------	--------	0.5 ml
1.5M Tris-Cl pH 8.8	0.387 ml	0.387 ml	-------
Glycerol	---------	0.560 ml	-------
ReadySol IEF 40% solution	0.154 ml	0.580 ml	0.180 ml
10% (w/v) SDS solution	0.016 ml	0.016 ml	0.02 ml
10% (w/v) APS solution	0.01 ml	0.005 ml	0.01 ml
TEMED	0.003 ml	0.0015 ml	0.004 ml
Total volume	1.55 ml	1.549 ml	2 ml
To give 1 cm stacking gel	1.55 ml per slab gel	1.55 ml per slab gel	2 ml per slab gel

Appendix 9 : PolyacrylamideGel and Polyacylamide-Gradient Gel including xylan and Urea-Gradient Gel Solutions

A. Polyacrylamide resolving gel(T10 C3.9) and polyacrylamide-gradient gel (3.97-14.97% T) including xylan

Reagents	Resolving gel (T10 C3.9)	Ligth solution (T3.75 C3.9)	Dense solution (T14.97 C3.9)
MilliQ water	4.7 ml	1.482 ml	---------
5%(w/v) xylan in water	0.2 ml	0.048 ml	0.048 ml
1.5M Tris-Cl pH 8.8	2.5 ml	0.6 ml	0.6 ml
Glycerol	------	-------	0.852 ml
ReadySol IEF 40% solution	2.5 ml	0.225 ml	0.9 ml
10% (w/v) SDS solution	0.1 ml	0.026 ml	0.026 ml
10% (w/v) APS solution	0.05 ml	0.015 ml	0.075 ml
TEMED	0.01 ml	0.005 ml	0.0025 ml
Total volume	10 ml	1.55 ml	1.549 ml
To give 1 cm stacking gel	3.1 ml per slab gel	1.55 ml per slab gel	1.55 ml per slab gel

B. Urea-gradient gel (0 to 6 M Urea) solutions

Reagents	Ligth solution (T10 C3.9),	Dense solution (T10 C3.9)	Stacking gel (T3.5 C3.9)
MilliQ water	1.217 ml	0.291 ml	1.335 ml
0.5M Tris-Cl pH 6.8	--------	--------	0.5 ml
1.5M Tris-Cl pH 8.8	0.6 ml	0.6 ml	-------
ReadySol IEF 40% solution	0.609 ml	0.609 ml	0.175 ml
Urea	--------	0.9 g	-------
10% (w/v) APS solution	0.0075 ml	0.0075 ml	0.02 ml
TEMED	0.0025 ml	0.0025 ml	0.008 ml
Total volume	2.436 ml	2.436 ml	2 ml
To give 1 cm stacking gel	1.50 ml per slab gel	1.50 ml per slab gel	2 ml per slab gel

Appendix 10 : Diamine and Nondiamine Silver Staining Methods

A. Ammoniacal silver solution (Diamine silver) accordingly with Oakley *at al.*(1980)

Step	Procedure	Time
1	Soak gel in 40%(v/v)ethanol, 10%(v/v)acetic acid	30 min
2	Rinsing 3 x 30 min in water	1.5 h
3	Sensitization in 10% (w/v) glutaraldehyde in water	30 min
4	Rinsing 4x15 min in water	1 h
5	Impregnation in silver solution prepared by mixing 1.4 ml conc NH_4OH to 21 ml of 0.36% (w/v) NaOH. Stir vigorously and slowly add 4 ml of 19.4% (w/v) $AgNO_3$. Make up to 100 ml and keep in dark bottle.	20 min
6	Rinsing 3x 5 min	15 min
7	Development in solution 0.005% (w/v) citric acid and 0.019% (w/v) formaldehyde	3-10 min
8	Termination by rinsing with 5% (v/v) acetic acid in 20% (v/v) ethanol	20 min
9	Storage in methanol 20% (v/v), 3% (v/v) glycerol before drying gel.	>1 h

B. Silver nitrate by Merril *et al.* (1981)

Step	Procedure	Time
1	Soak gel in 40%(v/v) ethanol, 10%(v/v)acetic acid	30 min
2	Second fixation, 2 x 15 min 10%(v/v) ethanol, 5%(v/v) acetic acid	30 min
3	Oxidaser, 0.128 g potassium dicromate, 31 µl conc HNO_3/ 100ml sol	5 min
4	Washing, 3x5 min or until clear gel in water	15 min
5	Impregnation, 0.2 g $AgNO_3$ / 100 ml water	20 min
6	Rinsing in water	1 min
7	Development in 6g $NaCO_3$, 100 µl formaldehyde /200ml water	30 sec
8	Developer (formaldehyde added just before using)	~5 min
9	Termination by rinsing with 5% (v/v) acetic acid in 20% (v/v) ethanol	20 min
10	Storage in methanol 20% (v/v), 3% (v/v) glycerol before drying gel.	>1 h

* All with the best quality water available.

Appendix 11 : Other Silver Staining Procedures

A. Blum *et al.* (1987)

Step	Procedure	Time
1	Soak gel in 40%(v/v)ethanol, 10%(v/v)acetic acid	30 min
2	Washing, 3x20 min 50% (v/v) ethanol	60 min
3	Soak sodium thiosulphate($Na_2S_2O_3$) 2 ml stock A / 200 ml water	1 min
4	Rinse 3 x 20 sec water	1 min
5	Impregnation, 2 ml stock B, 150 µl formaldehyde (37%)/200 ml water	20 min
6	Rinse 2 x 20 sec	40 sec
7	Develop: 20 ml 6% $NaCO_3$, 10 µl HCHO, 4µl $Na_2S_2O_3$/ 200 ml water	~7 min
8	Termination by rinsing with 5% (v/v) acetic acid in 20% (v/v) ethanol	20 min
9	Storage in methanol 20% (v/v), 3% (v/v) glycerol before drying gel.	>1 h

Stock A = 2%(w/v) $Na_2S_2O_3$ in water. Kept at 4°C in a dark bottle.
Stock B = 10%(w/v) $AgNO_3$ in water. Kept in a dark bottle.

B. Rabilloud *et al.* (1988)

Step	Procedure	Time
1	Soak gel in 30%(v/v)ethanol	30 min
2	Wash 2 x 15 min	30 min
3	Sensitizing 0.025 g sodium dithionite ($Na_2S_2O_4$) / 100 ml water	1 min
4	Rinse, 2x30 sec with water	1 min
5	Impregnation, 0.2 g $AgNO_3$, 8µl HCHO (37%) / 100 ml water	25 min
6	Rinsing in water	1 min
7	Develop: 6g $NaCO_3$, 48 µl HCHO, 20µl 2% $Na_2S_2O_3$/ 100 ml water	~7 min
9	Termination by rinsing with 5% (v/v) acetic acid in 20% (v/v) ethanol	20 min
10	Storage in methanol 20% (v/v), 3% (v/v) glycerol before drying gel.	>1 h

Note: Sensitizing with $Na_2S_2O_4$, developer with Thiosulphate ($Na_2S_2O_3$)

* All with the best quality water available.

Appendix 12 : Schiff's Reagent and Alcian Blue Pre-staining.

A. Preparation of Schiff's reagent

1. Heat 800 ml distilled water to almost boiling temperature.
2. Add 4g basic Fuchsin and stir for 5 minin a boiling water-bath.
3. Cool in an ice-bath to 50 °C (not below 40°C).
4. Add 80 ml of 1 M HCl. Mix, and cool to 25 °C.
5. Add 6.8 g of sodium *meta*-bisulphite. Mix, and let sit overnight at 4°C in the dark.
6. Add 20 g of charcoal. Mix, and let charcoal sediment. Filter the supernatant through a glass scintered Buchner funnel. The filtrate should be colourless or weakly pink. If red, add more charcoal and filter again.
7. Store in a dark bottle at 4 °C. The reagent is stable for at least a year.

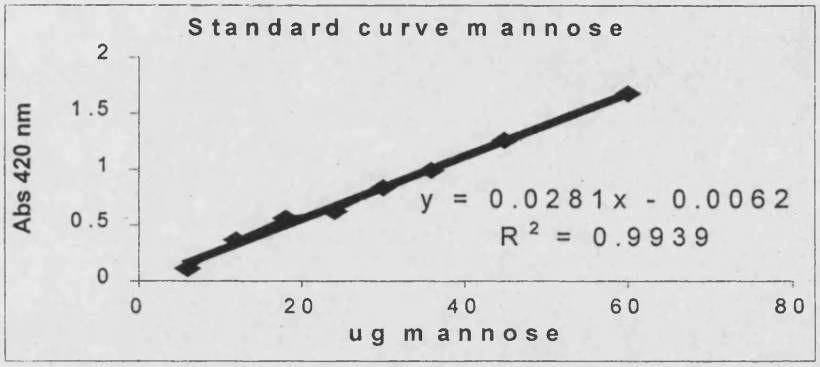
B. Alcian Blue pre-staining.

Step	Procedure	Time
1	Soak 3 x 10 min in 30%(v/v) ethanol	30 min
2	Wash 2 x 5 min in 5%(v/v) acetic acid	10 min
3	Oxidation in 1% (w/v) periodic acid in 5%(v/v) acetic acid	20 min
4	Rinse, 2x 2 min with 5%(v/v) acetic acid	4 min
5	Rinse, 2x 2 min with water	4 min
6	Soak, 0.5% (w/v) potassium metabisulphite in water	12 min
7	Rinse, 2x 2 min water at 50 °C	4 min
9	Soak, 2x 2min 25% (v/v) ethanol, 10% (v/v) acetic acid at 50°C	4 min
10	Stain, 0.125% Alcian Blue in 25% ethanol, 10% acetic acid at 50°C	15 min
11	Wash in 25% ethanol, 10% acetic acid at 50°C	1 min
12	Wash in 25% ethanol, 10% acetic acid at 50°C	4 min
13	Wash in 25% ethanol, 10% acetic acid at 50°C	8 min
	When gel is clear, proceed with silver staining	

* All with the best quality water available.

Appendix 13 : Standard Curves for Total Sugar and Sialic Acid Determination

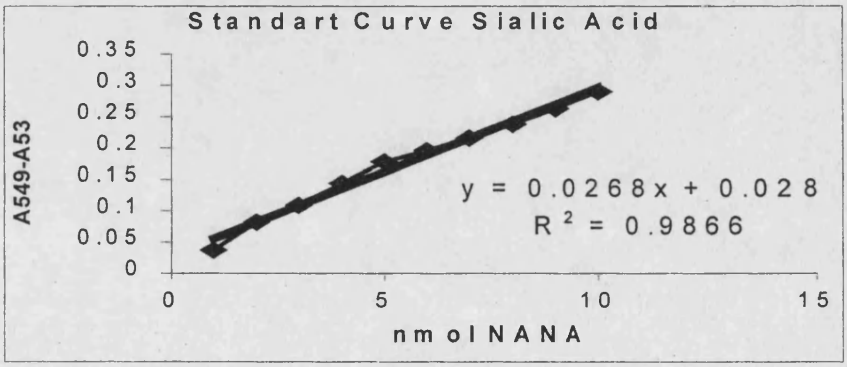
A. Standard curve for the determination of sugar content



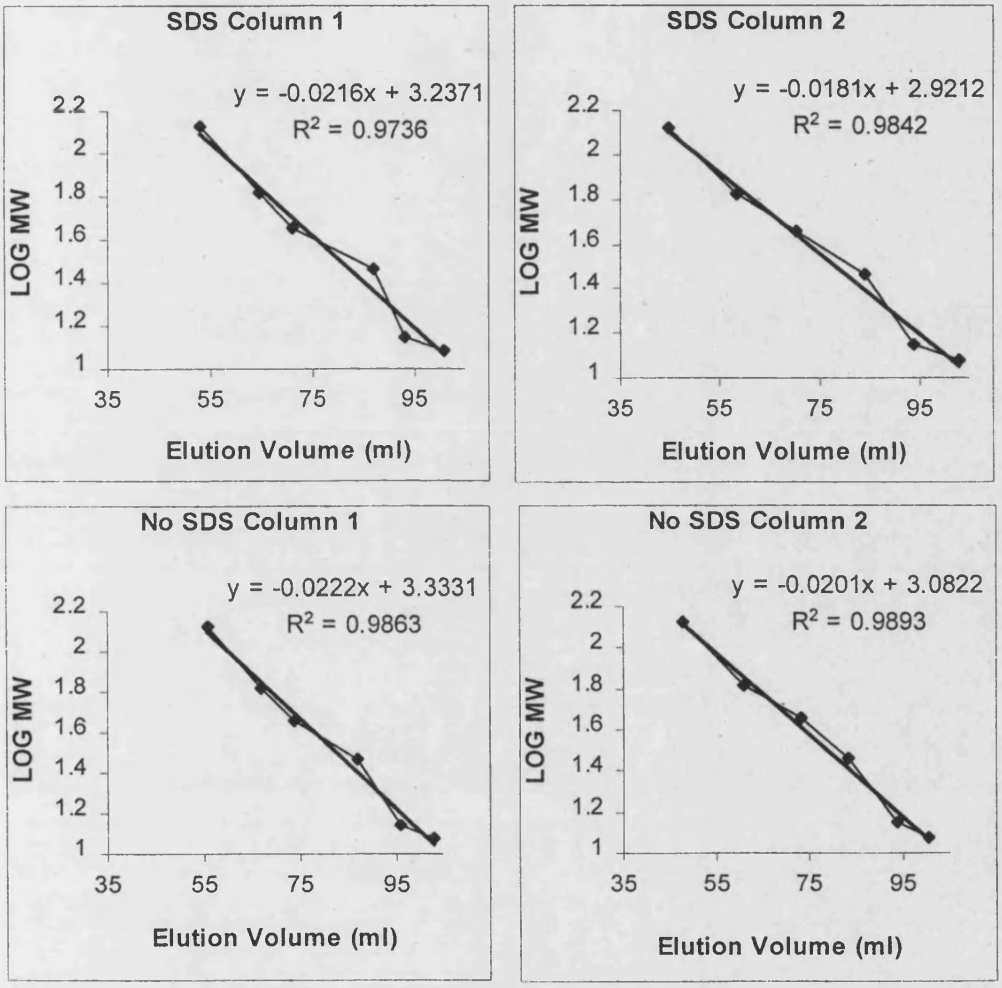
B. Reagents for the Thiobarbituric acid Assay for Determination of Sialic acid Content after Enzymatic Release and Purification as by Manzi and Varki (1993)

Periodate reagent	Dissolve 4.3 g of sodium *meta*-periodate in 4 ml water. Add 58 ml of concentrated *ortho*-phosphoric acid, and make up to 100 ml. Store at 4 °C in a dark bottle covered with foil (stable indefinitely).
Arsenite reagent	Dissolve 10.0 g of sodium arsenite and 7.1 g sodium sulphate in 0.2 M sulphuric acid and make up to 100 ml in the same acid. Store at room temperature (stable indefinitely).
TBA reagent	TBA reagent : dissolve 1.2 g of 2-thiobarbituric acid and 14.2 g sodium sulphate in water(complete dissolution is obtained by dropwise addition of 1 M NaOH solution) and make up to 200 ml. Store at room temperature (stable for several months).

C. Standard curve for the determination of Sialic acid

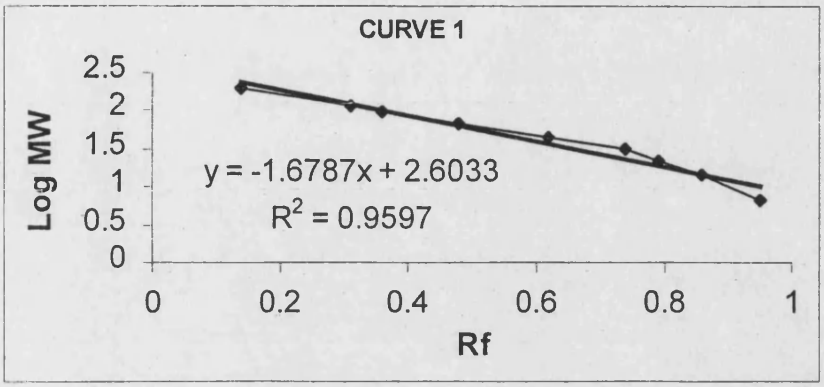


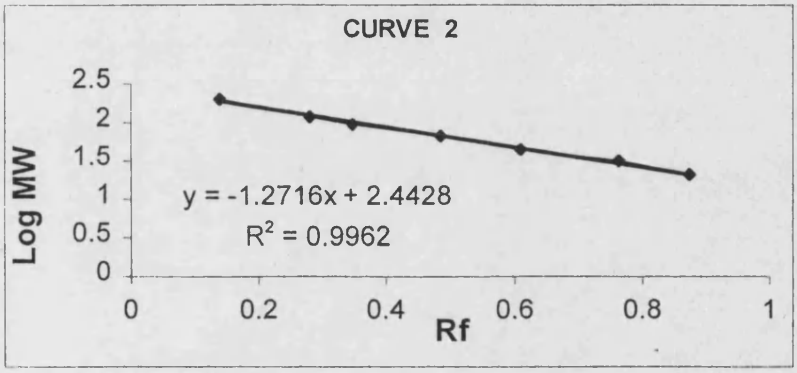
Appendix 14 : Standardisation of Gel Filtration Columns 1 and 2 using elution buffers with and without SDS and reducing agent

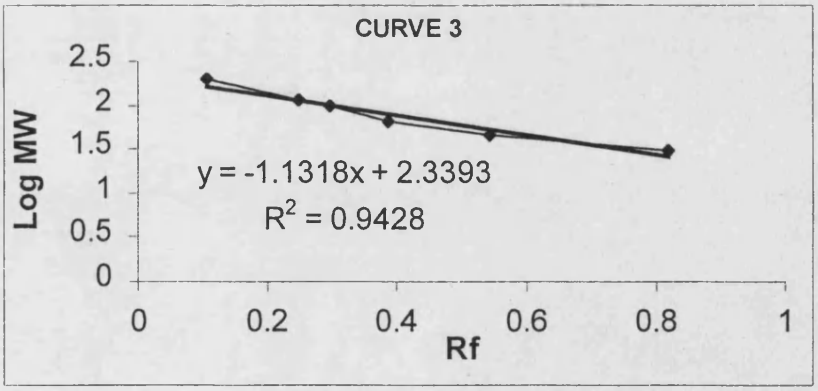


The purified proteins used were: Bovine Serum Albumin (66000 KDa), Egg Albumin (43000 KDa), Carbonic Anhydrase (29000 KDa), Lysozyme (14000 KDa) and Cytochrome c (12400 KDa).

Appendix 15 : Standard Curves for the Determination of Molecualr Weights of Unknown Proteins after SDS- PAGE.







Appendix 16 : LB and NYZ Media

A. LB Media

LB Broth	**l^{-1}**
Bacto Tryptone	10g
Bacto Yeast Extract	5g
NaCl	10g
Adjust pH 7.0 with NaOH	
LB Agar	**l^{-1}**
LB broth	1 l
Agar	20g
Adjust pH 7.0 with NaOH, Autoclave and pour into petri dishes.	
LB Top Agar	**l^{-1}**
LB broth	1 l
Agarose	7.0g
Adjust pH 7.0 with NaOH. Autoclave	

B. NYZ Media

NYZ Broth	**l^{-1}**
NaCl	5.0g
$MgSO_4.7H_2O$	2.0g
Yeast extract	5.0g
NZ amine (casein hydrolysate)	10g
Adjust pH to 7.5 with NaOH. Autoclave	
NZY Agar	**l^{-1}**
NZY broth	1 l
Agar	15 g
Adjust pH to 7.5 with NaOH. Autoclave and pour into petri dishes	
NZY Top Agar	**l^{-1}**
NZY broth	1 l
Agarose	7 g
Adjust pH to 7.5 with NaOH. Autoclave	

Appendix 17 : Agarose Gel Electrophoresis and Restriction Digestion of DNA

A. Efficient Range of Separation of Linear DNA by Various Gel Concentrations.

Agarose gel concentration (%)	Efficient range of separation of linear DNA (kb)
0.3	60 - 5
0.5	25 - 1.5
0.7	10 - 0.8
1.0	7 - 0.5
1.2	6 - 0.4
1.5	3 - 0.2
2.0	2 - 0.1

B. DNA Electrophoresis buffers

Tris-acetate (TAE) 50X concentrated stock solution	242g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)

Tris-borate (TBA) 10X concentrated stock solution	108 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)

C. Typical reaction mixture for Restriction Digestion of DNA

10X Digestion Buffer	2 µl
DNA solution	10 µl (containing 10-20µg DNA)
Restriction enzyme	1 µl
Sterile MQ-water	7 µl
Total volume	20 µl

Appendix 18 : Preparation of Hybridisation Solution and 10x SSC

A. Stock Solutions

Sodium Phosphate (Na_2HPO_4) 500mM	134g / 1000 ml. Adjust pH 7.2 with H_3PO_4
Ethylenediaminetetra-acetic acid disodium salt (EDTA Na_2) 500mM	186.12g / 1000 ml. Adjust pH 8.0 with NaOH
Polyethyleneglycol 600 (PEG -600) 40%	200g / 500 ml.
Sodium dodecyl sulphate (SDS) 20%	200 g / 1000ml.

B. Preparation

Na_2HPO_4 500mM pH 7.2	175 ml
EDTA $Na_2$500mM	1 ml
PEG -600 40%	100 ml
SDS 20%	175 ml

C. 10x SSC

NaCl	87.65 g
Sodium citrate	44.1 g
Deionised water	800 ml

Adjust pH to 7.0 with few drops 10 M NaCl

Add deionised ised water to a final volume of 1 litre

"'Potential', 'Probable', 'By Similarity' -
They don't know anything about this protein!"